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Reports of ring-tests on the Honeybee (Apris mellifera) homing flight test following single exposure to sublethal doses of test chemical, conducted between 2015 and 2019

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Foreword

The ring-test reports presented in this document summarise a multi-year project (2015-2019) led by France to internationally validate the homing flight test in honeybee (Apis mellifera L.) after single exposure to sublethal doses of a test chemical. The reports were were prepared by Julie Fourrier from the French Scientifique and Technical Institute for Beekeeping and Pollinisation (in French: Institut technique et scientifique de l'apiculture et de la pollinisation). Each report provides details on the material and method, the test design and procedures, the participating laboratories, the test results and analysis of test data and conclusions.

Following these studies, an OECD guidance document was developed in 2020 presenting the optimal test design proposed for this field study that evaluates the homing success of honeybees after single exposure to sub-lethal doses of a test chemical. The Guidance document was approved by the Working Party of the National Coordinators of the Test Guidelines Programme in April 2021, and is published as No. 332 in the OECD Series on Testing and Assessment.

The Guidance Document is published under the responsibility of the Chemicals and Biotechnology Committee.

Part I - Final report: Summary of the Results of the First international ring test 2015 for the standardisation of an homing flight test design

Final report
Summary of the Results of the First international ring test 2015 for the standardisation of an homing flight test design

Homing flight test in honeybee (*Apis mellifera* L.) after single exposure to sublethal doses

Compiled by
Julie Fourrier

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11 December 2015

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1-INTRODUCTION

The homing test proposes to assess the effects of a single and oral exposure to sublethal doses of a chemical (technical grade active substance or a formulation) administered in controlled conditions on the homing performances of forager bees in the field. The success of the homing flight in exposed foragers versus non-exposed foragers is measured over the short term.

The validation phase of the methodology was initiated in 2014 in French laboratories ITSAP/ACTA and INRA Le Magneraud. We conducted experiments according to the methodology of Henry et al. (2012) with the test substance thiamethoxam at the sublethal dose of 1 ng per bee. This dose corresponds to a NOEL ('No Observed Effect Level') on mortality 48-h after exposure. The homing failure was determined from an individual versus a collective exposure, per group of 10 foragers' bees, to choose the standardized mode of administration. Our results were similar to those of Henry et al. (2012). For both individual and collective administration of thiamethoxam to bees, exposed honeybees returned to the hive in significantly lower proportions than controls (**Appendix 1**). Moreover, for both control and exposed treatments, honeybees exposed individually returned to the hive in the same proportions than honeybees exposed in groups. So, we retained the collective exposure, easier to perform (**Appendix 1**).

In 2015, a first international based ring test with willing labs from OECD member countries was performed. Eleven European laboratories from Germany, United Kingdom, Switzerland, Italy, and France participated to the ring test. Ten laboratories could conduct the test as two laboratories worked together. After a first meeting and practical formation session to the methodology in April 2015, the ring test was performed from June to August 2015. The test endpoint was the determination of a No-Observed-Effects-Dose (NOED) on the homing flight.

We used the active substance thiamethoxam as a reference item in this ring test. We tested three sublethal doses of the molecule according to a geometric progression with a ratio of 3: 1 ng, 0.33 ng and 0.11 ng a.i. per bee. A control (acetone 0.1 % in a 30 % w/v sucrose solution) was included. All the labs used technical grade thiamethoxam with the same batch number (purity = 99%).
For each test replicate, bees were collectively exposed per group of bees to test item treatments or to the control.

We worked with foragers bees familiar with their environment thanks to the plantation of a Phacelia plot. Indeed, foragers carrying purple-bright pellets of Phacelia pollen are easily recognizable. This ensures that bees knew the Phacelia plot. Phacelia was planted at 1 km (+/- 100 m) of the experimental colonies in order to test the effect of the sublethal doses of the reference item on the homing flight of foragers bees released at a long distance (Henry et al. 2012). This distance is in the range of those covered by foragers during normal foraging flights (Steffan-Dewenter and Kuhn, 2003)
The homing flight was recorded in the field during a 24-h period after bee release using RFID technology. Three replicates of the homing test had to be performed, each one with a different colony, in order to determine a No-Observed-Effects-Dose (NOED) on the homing flight as the endpoint value. For each treatment, a homing rate and its duration 24h after release were calculated. The range of variability in the homing success results was also established for each replicate and laboratory. Homing flight success obtained for control bees were considered to set a validity criterion.

In parallel to the homing flight ring test 2015, additional experiments were conducted in ACTA/ITSAP and INRA Le Magneraud laboratories. One experiment aimed to assess acetone effects on homing flight when used as a test control and the other experiment was used to establish a possible alternative to the plantation of a Phacelia field identified as a possible limiting point for the test performance.

2-INFORMATION ON THE RING TEST GROUP

Eleven laboratories participated to the ring test 2015. But, two laboratories worked together on the ring test (CRA-API and Biotecnologie BT S.r.l). Therefore, 10 laboratories could conduct the test.

Laboratory	**Responsible person(s)**
ITSAP-Institut de l'Abeille, **France** ***Leader of the ring test group*** ***Ring test organisation***	Julie Fourrier (ACTA and ITSAP)
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BioChem agrar GmbH, **Germany**	Markus Barth Melanie Hänsel
LAVES Institute for Apidology Celle, **Germany**	Martina Janke Dorothee Lueken
TESTAPI, **France**	Hervé Giffard Claire Molitor

Organisation and coordination of this work was supported by grants from the French Ministry of Agriculture (FranceAgriMer) and Lune de Miel® Foundation.

3-RING TEST SCHEDULE

Phacelia sowing	End of March to May 2015
Ring test meeting and practical formation	April, 14 to 16, 2015
Experimental period	June to August 2015 with Phacelia blooming
Delivery of results	July to October 2015
Analyses and evaluation of the ring test results	July to December 2015

4-MATERIAL AND METHODS

4.1 Crop

Phacelia: a plot of Phacelia approximately 0.5 to 1 hectare in size was sowed so that the nearest border is 1 km (+/- 100 m) away from the experimental colonies. The length of the shortest plot edge had not to be less than 30 m. Due to its specific pollen, this plant allowed us to identify foragers of interest. No chemical treatment was realized on phacelia plot until the end of the test including seed dressing and soil treatment.

There should be no other plot of blooming Phacelia within 4 km around the experimental hive (about twice the average distance covered by the pollen foragers in a simple agricultural landscape, according to Steffan-Dewenter and Kuhn, 2003). High density of hive (more than 50 colonies) in a 1-km radius away from phacelia plot should be avoided.

Start of the experiments: it was recommended to start the experiments with at least 50% of the plot of blooming Phacelia, but it was possible to begin shortly before if the bees already foraged Phacelia field.

4.2 Honeybees

Source of the colonies, treatments and health status: Chemical treatments (anti-varroa…) should have been completed at least 4 weeks before the start of the experiment. Healthy colonies (as far as possible disease-free) and queen with known history and not older than 2 years, were used.

Hives characteristics: Ten to twelve frames hives were used. To be sure that bees will correctly circulate through RFID readers to get in and out of the hive, , hives with a full floor (no mesh) if cool summer temperatures were encountered, or a system consisting of two mesh floors fixed together (or two half mesh floors placed in a staggered configuration) and separated by few centimetres were used. According to climatic conditions, one to two supers could be added to increase hive volume and good thermoregulation during summer climatic conditions.

Preparation of the colonies: The test colonies used for this ring test were homogenous regarding colony strength, food storage, brood and preparation. They were of at least 10,000 honeybees. For hives with ten frames configuration, colonies comprised 4 to 7 brood combs (uncapped larvae and nymphae) for hives with ten frames configuration and 6 to 8 brood combs for hives with twelve frames configuration, 2 to 3 food combs and at least 1 empty

frame. A complete apiarist visit was performed for each tested colony one or two days before the experiments to prepare colony and to verify health status.

Installation of the colonies: All the colonies used for the test were installed on the experimental site, 1 km away from the Phacelia plot, at least 4 days before the start of the test to allow knowledge of environment by honeybees. The 3 colonies had to be separated from few meters (≥ 4 meters). The presence of foragers carrying Phacelia pollen coming from the plot implanted was checked at the entrance of the hives prior to the experiment.

4.3 RFID device

RFID (Radio Frequency Identification) device: The RFID technology (Streit et al. 2003; Decourtye et al. 2011) allows detecting each time a tag-equipped bee was passing in nearness of a reader (working distance of 3 mm). The principle depends on the emission of a radio signal emitted by the reader which is received by the tag positioned on the bee. The tag is not equipped with a power source (passive function) and it obtains its operating power from the reading process to emit a unique identification code. Reader automatically recognizes a virtually unlimited number of individual insects and the reading can cross physical barriers (propolis, wood, plastic and dust).

We used RFID tags (13.56 MHz system; Microsensys GmbH, Erfurt, Germany). Tags were of 1 mm wide by approximately 2 mm long. They weighed no more than 5 mg, equivalent to approximately 5% of the weight of a worker bee, which was significantly 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). RFID microchips were glued on the thorax of the bees.

RFID system used was MAJA system (Microsensys GmbH, Erfurt, Germany). It comprised one Host (small computer with Windows system) that recorded data connected to the readers. Four readers were placed at the entrance of the hive (parallel arrangement). Each reader spanned a tunnel of 14 × 21.5 mm (7 mm high). Readers were installed at the hive entrance thanks to an interface (in plastic or wood) between hive and readers (= mask). Then, the bees entered into the hive through 4 possible entrances associated with readers.

The tag identification code and the exact time of the event (date, hour, minute and second) were recorded with the MAJA Host capture software. RFID data were collected by connecting MAJA Host with a PC laptop equipped with Microsoft Mobile Device Center software thanks to an USB cable. Time events (date, hour, minute and second) between MAJA Host and PC laptop had to be synchronized before data collection.

Reading rates: They should be known and covered at least 95% of the crossing of bees. To achieve this, measures were taken – before the system is fitted to the hive, or not – by simulating honeybee crossing with microchips glued on small plastic or wood sticks.

Hives equipment: As soon as the colonies were installed on the experimental site, 1 km away from the plot of Phacelia (**four days before the test**), one hive was equipped with the RFID device. For at least one other colony tested after, a mask (or blank) that mimics RFID system was placed at the hive entrance already to allow the knowledge of equipped hive entrance by the foragers before experiments.

Tags Batches: Honeybee identification with RFID microchip code was the object of a dataset established before the experiment. This dataset of microchips number classified the honeybees per treatment modality in a way to allocate them to the corresponding treatment after their homing flight to the hive. Three to four batches of 10 to 15 tags (bees) were prepared per each replicate and treatment.

4.4 Test item

Test item : Technical grade neonicotinoid active ingredient (a.i.) thiamethoxam Supplier : Laboratories Dr. Ehrenstorfer-Schäfers, Augsburg	Batch number : 31119 Appearance/colour: crystalline solid/colourless Intended use : insecticide
CAS number : 153719-23-4 Chemical formula of a.i. : C8H10CIN5O3S	Purity : 99.0% Water Content : 0.0%
Date of analysis : 15.01.2014 Analysis laboratory : Labor Dr. Ehrenstorfer-Schäfers Analytical method : HPLC/DAD Risk symbol (s): Xn, N Expiry date: 19.11.2017	Storage conditions : ambient temperature (20°C ± 4°C) ventilated room protected from frost Precautions: appropriate precautions of hygiene and security, according to the safety data sheet

All the labs used technical grade thiamethoxam with the same batch number. Certificate of analysis is proposed in **Appendix 2**.

4.5 Test design

Number of treatments:	1 control group and 3 test item groups
Number of bees labelled and exposed per treatment and replicate:	minimum of 30 bees => 3 cages of 10 tested bees respectively for 30 bees tested (the cage is the experimental unit)
Number of test replicates:	3, each one with a different colony

4.6 Preparation of the test item and test feeding solutions

The preparation of the test item and test feeding solutions is presented in **Appendix 3**.

Test item solutions: A stock solution of the test item solutions were prepared and could be stored in the refrigerator at 4 °C ± 4 °C up to 5 days before the test. Acetone was used as the solvent. A control with acetone solvent was prepared (acetone ≥ 98%).

Test feeding solutions: The test item and control solutions were administered in a sucrose feeding solution containing 30% (w/v, sucrose/demineralised water). In proportion, this corresponded to 30 g of sucrose in 100 ml of demineralised water.
The final volume of the test or control solutions in the 30% (w/v) sucrose feeding solution was 0.1% (v/v). Test feeding solutions prepared could be stored at 4 °C ± 4 °C up to 1 day before the test.
The test item or control solutions as well as the sucrose solutions were changed for each test replicate, and were stored in a deep freeze at –20°C ± 2°C after the test for the concentration

and dose of the tested chemical to be analytically determined. To do so, the 3 treated test feeding solutions (≥ 5 ml of solution per sample) for the 3 test replicates conducted were centralized at ITSAP/ACTA laboratory before being sent to the French food safety agency (ANSES, Sophia Antipolis) for analytical analyses.

4.7 Test cages

The cages should be of suitable size to the number of the foragers captured and well-ventilated. They could also have two transparent sliding walls for the manipulation and observations of honeybees in laboratory and to facilitate release phase in the Phacelia field after treatment.

4.8 Homing flight test procedure

Bees capture at the hive entrance

Foragers captured: Only foragers carrying **pellets of Phacelia pollen,** coming from the Phacelia plot specifically sowed, are captured at the entrance of the hive in the morning on the flight board and grouped into cages with food *ad libitum*. Candy or sucrose solution (50 % w/v sucrose/ demineralised water) were used. Water could be brought once the bees are captured. Cages with collected bees were then kept in an isolated box (e.g. cooler). A wet towel could be put inside the box in order to avoid overheating or dry up.

Number of foragers captured: A sufficient number of foragers had to be captured to obtain at least 30 foragers honeybee labelled with a RFID microchip per treatment and per replicate after exposure to the test feeding solutions. For this, a capture of at least 140 foragers was recommended.

If not enough foragers carrying pellets of Phacelia pollen were captured the day of the test, then the already captured bees were labelled, exposed in laboratory and released in the field but the day after, another test day (capture, label and release) was conducted to have the number of bees required for the replicate concerned (minimum of 30 foragers bees labelled per replicate and treatment). Then in this case, two successive test days were conducted for the replicate.

Labelling and exposure in the laboratory

Starvation and labelling phase: The captured foragers were brought to the laboratory (25 ± 2°C). The bees underwent a 1.5 to 2 hours starvation period. During this period, the bees were transferred one by one from cages to a holding cage where a foam plunger allowed them to be immobilised without damage and labelled with a RFID microchip (e.g. queen marking device). The microchip was glued on the thorax of the foragers using glue such as “dental cement”. The glue is non-corrosive and dries very quickly (less than 2 minutes). During labelling phase, glue equipment was placed in crushed ice when not used to avoid the glue to dry immediately in the tip. The labelling was performed without using anaesthetic on the bees.
The RFID microchips were recorded and distributed per treatment beforehand (cf. 4.3 RFID device).
After labelling, the foragers were transferred in groups of bees (minimum of 3 cages of 10 bees per treatment) in the dark, before being exposed to the test item or to the control.

According to number of bees captured and labelled, number of bees per cage could vary from 6 to 15 bees. The cages prepared with labelled bees were placed in the dark waiting for exposure phase.

Exposure phase:
Test item doses: **0.11, 0.33 and 1 ng a.i per bee (geometric progression with a ratio of 3)**. The highest dose corresponded to a NOEL ('No Observed Effect Level') on mortality 48-h after exposure.

Application procedure: The honeybees were exposed by feeding them with 20 µl per honeybee (200 µl per group of 10 bees) of the 30% (w/v) sucrose solution containing the test item at different doses or the control solution. The volume of sucrose solution is distributed using a feeder system enabling contact with the food only through the mouth parts (for example: the tip of a micropipette beveled). The bees in a cage shared the feeding solution by trophallaxis. The exposure phase is completed once the honeybees have consumed all the administered volume.

Application conditions: Maximum exposure duration was an hour and a half. The start and end time of exposure are recorded for each cage. Sucrose solution consumption was regularly monitored. If bees from some cages didn't consume all the sucrose solution volume, then the feeders were weighed in order to further calculate the real dose received per bee. Feeders were weighed empty and full beforehand.

Post-application: The cages of honeybees which ingested all the administered feeding solution underwent an additional one hour starvation period in the dark.
Before releasing the bees, the dead honeybees and those that may have lost their microchips were recorded. They were collected during release phase to be identified thanks to microchip code and to be removed from released labelled bees.

Honeybees release

Transport: The honeybees were transported to the edge of the plot of Phacelia, 1 km (+/- 100 m) from the hive equipped with the RFID system to be released. Temperature and humidity levels during transport should ensure their safe keeping, particularly if the place of release is a long way from the laboratory (transport in cool boxes containing a damp cloth, in a box incubator…)
If the experimental site was not at the same place than the laboratory and implied time of transport before bee release, this one was included in the one-hour starvation period after exposure.

Release: The cages representing the tested treatments were put in the same place, on a flat surface at least 100 cm off the ground, and then opened simultaneously. If necessary, the bees were emptied out. At least two hours were considered between the release and nightfall to allow foragers flight to the hive. Release start and end time (hour and minutes) were recorded. A thermo-hygrometer or other suitable device allowed punctual weather conditions (temperature and hygrometry (%)) to be measured during the release phase.

Climatic conditions: Weather conditions should be favorable to foraging (wind below 5 of Beaufort scale, temperatures of at least 15°C and no rain) when the honeybees were released.

4.9 Test Schedule

Test schedule: The capture, labelling, exposure and release phases for the test took place over one day. In the event that the number of honeybees captured at the hive was not sufficient to release enough honeybees per tested treatment (at least 30 bees per treatment and replicate), the test could take place over two consecutive days in order to increase the number of honeybees released.

The RFID recordings of the labelled foragers' homing flight to the hive started immediately after the release and lasted 24 hours per replicate (two consecutive 24-hours periods of recording if two consecutive test days were performed for a replicate). This 24h-recording period has previously shown to be relevant to record the homing success of released bees (Henry et al. 2012, French laboratories results 2014).

4.10 Assessment

The data recorded with RFID readers for the bees returning to the hive: the microchip number, the reader number and the read time (date, hour, minute and second). These raw data were recorded in electronic form in the MAJA Host storage system equipped with the appropriate software thanks to a PC connected to the host. Recorded raw data were collected 24 hours after the release. They could be collected 48 hours after release if two days were necessary to complete the test replicate.

The weather conditions: temperature (T°C) and hygrometry (%) per hour were recorded thanks to a data logger given to each laboratory and placed under the tested hive with RFID system. Rainfall (mm) per day was also recorded at the same place with a rain gauge.

4.11 Results presentation and statistical analysis

The number of dead bees after labelling and before release in the field was used to calculate a mortality rate per treatment for each replicate.

The homing flight was characterised by two variables to explain depending on applied treatments:

- The homing flight success (**main variable**), which is a binomial variable with a value of 1 if the honeybee returns to the hive over the 24-hours period, or 0 if it does not return.
- The homing time 24 hours after release (**secondary variable**), which is a quantitative variable. For each honeybee, it is defined as the time between the release and the first recording at the hive.

The success of the homing flight and its duration was determined from three files: one from honeybees released (microchips code), one from information at the release station (date, hour and minutes of release) and the other one from the RFID recording at the hive. The three files provided one raw data file per identified honeybee and treatment where homing time was expressed in minutes. During the 24 hours of RFID recording, a same honeybee can be recorded several times when it goes in or out the hive for foraging activities. Then, several homing time can be calculated for a same bee. We only kept the lower homing time per bee which corresponds to the first recording at the hive after release. A bee that doesn't return to

the hive after release is indicated in the raw data file thanks to microchip code, but there is no homing time calculated.

One raw data file was created per replicate and for the 3 replicates pooled together for data analysis. Data from the 3 test replicates are pooled to maximize total number of bees per treatment (total of ≥ 90 bees labelled with a RFID microchip) for the homing test analyses including data structuration and statistical treatments (Henry et al. 2012, EFSA, 2013 a, 2013 b). The results are presented as cumulative homing probability to the hive over the 24-hours period per test item treatment and control group.
Statistical analyses were performed using the statistical software R version 3.0.1.(From the results of the three test replicates (pooled data), the homing rates to the hive obtained over the 24-hours period for each treatment were compared using an exact binomial test ($P < 0.05$). An adjusted significance threshold was applied for paired comparisons with Bonferroni method. Concerning homing durations, data normality and homogeneity of variance were first tested with a Shapiro-Wilk test and a Bartlett test respectively ($P > 0.05$). As data didn't show normal distribution and/or variance homogeneity, homing durations obtained were compared between treatments using a non-parametric Kruskal-Wallis test ($P < 0.05$).

From the test data analyses, we determined a 'No Observed Effect Dose' on the homing flight. The NOED was expressed as ng of the test item per honeybee.

4.12 Validity criterion of the study

It was decided that the validity criterion of the test defined as the minimum homing rate acceptable for control honeybees would be set from the results obtained during the ring test. For that, we considered the homing success for the control bees for each replicate.

5-RESULTS AND DISCUSSION

Seven laboratories out of 10 performed completely the test. Two laboratories couldn't conduct the test and one laboratory (Laboratory 3) conducted only one test replicate because of problems with the Phacelia field (foraging competition between blooming Phacelia field and other blooming crops, competition with weeds and other pollinators within the Phacelia field, problems of Phacelia dryness because of summer climatic conditions).

5.1 Mortality in laboratory

The bee mortality was considered from the end of the labelling phase to the release phase in the Phacelia field.

Table 1: Labelled bee mortality before release in Phacelia field for test replicate 1

Lab	Bee race	Bees labelled and released	Control	Thiam. 0.11 ng/bee	Thiam. 0.33 ng/bee	Thiam. 1 ng/bee
1	Carnica x Buckfast	Nb of labelled bees	40	40	40	40
		Mortality (%)	0	0	0	0
		Nb of bees not released*	1	0	0	2
		Nb of released bees	39	40	40	38
2	Carnica	Nb of labelled bees	42	42	42	42
		Mortality (%)	9,5	4,8	16,7	9,5
		Nb of bees not released*	5	4	8	4
		Nb of released bees	37	38	34	38
3	Carnica	Nb of labelled bees	40	40	40	40
		Mortality (%)	0	0	0	0
		Nb of bees not released*	1	0	2	1
		Nb of released bees	39	40	38	39
4	Buckfast	Nb of labelled bees	30	30	30	30
		Mortality (%)	0	6,7	26,7	3,3
		Nb of bees not released*	0	2	8	1
		Nb of released bees	30	28	22	29
5	Ligustica	Nb of labelled bees	30	30	30	30
		Mortality (%)	0	0	0	3,3
		Nb of bees not released*	0	0	0	1
		Nb of released bees	30	30	30	29
6	Carnica	Nb of labelled bees	30	30	30	30
		Mortality (%)	0	0	0	16,7
		Nb of bees not released*	2	0	0	5
		Nb of released bees	28	30	30	25
7	Carnica	Nb of labelled bees	42	42	39	39
		Mortality (%)	0	0	0	0
		Nb of bees not released*	1	3	1	1
		Nb of released bees	41	39	38	38
8	Buckfast	Nb of labelled bees	35	35	35	35
		Mortality (%)	0	0	7,5	0
		Nb of bees not released*	4	1	6	1
		Nb of released bees	31	34	29	34

* Bees not released because of mortality, tags lost…

Table 2: Labelled bee mortality before release in Phacelia field for test replicate 2

Lab	Bee race	Bees labelled and released	Control	Thiam. 0.11 ng/bee	Thiam. 0.33 ng/bee	Thiam. 1 ng/bee
1	Carnica x Buckfast	Nb of labelled bees	40	40	40	40
		Mortality (%)	0	0	2.5	7.5
		Nb of bees not released*	1	0	1	3
		Nb of released bees	39	40	39	37
2	Carnica	Nb of labelled bees	39	40	43	42
		Mortality (%)	0	0	0	0
		Nb of bees not released*	1	1	0	0
		Nb of released bees	38	39	43	42
4	Buckfast	Nb of labelled bees	30	30	30	30
		Mortality (%)	0	0	0	0
		Nb of bees not released*	0	0	0	0
		Nb of released bees	30	30	30	30
5	Ligustica	Nb of labelled bees	30	30	30	30
		Mortality (%)	0	6,7	13,3	20
		Nb of bees not released*	2	8	13	10
		Nb of released bees	28	22	17	20
6	Carnica	Nb of labelled bees	30	30	30	30
		Mortality (%)	10	3,3	0	10
		Nb of bees not released*	3	1	0	3
		Nb of released bees	27	29	30	27
7	Carnica	Nb of labelled bees	42	42	39	39
		Mortality (%)	0	0	0	0
		Nb of bees not released*	1	0	2	3
		Nb of released bees	41	42	37	36
8	Buckfast	Nb of labelled bees	30	30	30	30
		Mortality (%)	0	0	2,5	0
		Nb of bees not released*	1	1	2	3
		Nb of released bees	29	29	28	27

* Bees not released because of mortality, tags lost…

Table 3: Labelled bee mortality before release in Phacelia field for test replicate 3

Lab	Bee race	Bees labelled and released	Control	Thiam. 0.11 ng/bee	Thiam. 0.33 ng/bee	Thiam. 1 ng/bee
1	Carnica x Buckfast	Nb of labelled bees	40	40	40	40
		Mortality (%)	5	5	15	7.5
		Nb of bees not released*	3	3	6	3
		Nb of released bees	37	37	34	37
2	Carnica	Nb of labelled bees	42	41	42	41
		Mortality (%)	4.8	5.1	19	14,8
		Nb of bees not released*	2	2	8	7
		Nb of released bees	40	39	34	34
4	Buckfast	Nb of labelled bees	30	30	30	30
		Mortality (%)	0	0	0	0
		Nb of bees not released*	0	0	0	0
		Nb of released bees	30	30	30	30
5	Ligustica	Nb of labelled bees	31	30	30	30
		Mortality (%)	3,3	0	0	0
		Nb of bees not released*	1	1	2	1
		Nb of released bees	30	29	28	29
6	Carnica	Nb of labelled bees	30	30	30	30
		Mortality (%)	6,7	3,3	6,7	3,3
		Nb of bees not released*	2	1	2	1
		Nb of released bees	28	29	28	29
7	Carnica	Nb of labelled bees	42	42	39	39
		Mortality (%)	0	0	0	0
		Nb of bees not released*	0	1	3	2
		Nb of released bees	42	41	36	37
8	Buckfast	Nb of labelled bees	40	40	40	40
		Mortality (%)	2,5	0	0	5
		Nb of bees not released*	4	2	0	2
		Nb of released bees	36	38	40	38

* Bees not released because of mortality, tags lost…

The volumes of treated sucrose solution administered to groups of bees during exposure phase were completely consumed each time, for each treatment and replicate and for all the laboratories. For the three replicates, mortality rate before release ranged from 0 to 26.7% (Tables 1 to 3). We note that mortality rates equal or higher than 19% were found three times, each time with a different treatment test and a different laboratory (laboratories 2, 4, 5). The laboratory 2 experienced as whole mortality for test replicate 1 and 3 whereas no problem of mortality was experienced for replicate 2. Differences in colonies sensitivity might be a possible cause. But mortality experienced did not impact homing flight performances and good results were recorded for the 3 test replicates (Table 7). The laboratory 4 experienced a mortality rate of 26.7% for bees exposed to 0.33 ng per bee of test item in first test replicate, but this also didn't impact homing results compared to homing results of replicates 2 and 3 for which no mortalities were recorded in the laboratory (Table 7). The laboratory 5 experienced as whole problems with exposure conditions in laboratory. For replicates 1 and 3, bees were fed ad-libitum with sucrose solution during post-exposure starvation period to avoid mortality. The laboratory 5 performed one hour starvation period after exposure for replicate 2 where mortality was experienced.

5.2 Homing success and homing duration per treatment

Results of the seven laboratories that could conduct the 3 test replicates are presented. Bees exposed to the highest dose of thiamethoxam at 1 ng per bee returned to the hive in significantly lower proportions than the bees exposed to control and to one or the two other tested treatments (exact binomial tests; $P < 0.05$; Table 4, A to G and Figure 1). Homing success didn't significantly differ between groups of control bees and groups of bees exposed to 0.11 ng and 0.33 a.i ng per bee of thiamethoxam (exact binomial tests; $P > 0.05$; Table 4, A to G and Figure 1). From the results of each laboratory, a common NOED of 0.33 ng per bee (nominal dose) on the homing flight was so determined.

Homing duration estimated 24 hours after release didn't significantly differed between groups of bees (Kruskal-Wallis tests; $P > 0.05$; Table 4, A to G). Some bees have shown to return relatively later to their colony even if a large majority of the bees come back during the 24 hours period after release. This was particularly observed for laboratories 1, 5, 6, 7 and 8 (Figure 2).
Details of statistical analyses for homing success and homing duration for each laboratory are presented in **Appendix 4**.

Table 4: Homing flight results for the ring test 2015 **(A to G)**
Results of the three test replicates pooled together for Laboratory 1 (A) to 8 (G) are presented.

A) Laboratory 1

	Control	**Thiam. 0.11 ng/bee**	**Thiam. 0.33 ng/bee**	**Thiam. 1 ng/bee**
Number of foragers released	115	117	113	112
Homing success probability (24 h after release) [1]	**0.661 (a)**	**0.692 (a)**	**0.726 (a)**	**0.268 (b)**
Result of Kruskal-Wallis test on homing duration (24 h after release)	**H = 4.973, df = 3, P = 0.174**			

[1]Pairwise comparisons were performed with binomial tests and used Bonferroni P value adjustment method. Same letters indicate no significant differences.

B) Laboratory 2

	Control	**Thiam. 0.11 ng/bee**	**Thiam. 0.33 ng/bee**	**Thiam. 1 ng/bee**
Number of foragers released	115	116	111	114
Homing success probability (24 h after release) [1]	**0.913 (a)**	**0.922 (ab)**	**0.874 (ac)**	**0.807 (c)**
Result of Kruskal-Wallis test on homing duration (24 h after release)	**H = 7.023, df = 3, P = 0.071**			

[1]Pairwise comparisons were performed with binomial tests and used Bonferroni P value adjustment method. Same letters indicate no significant differences.

C) Laboratory 4

	Control	**Thiam. 0.11 ng/bee**	**Thiam. 0.33 ng/bee**	**Thiam. 1 ng/bee**
Number of foragers released	90	88	82	89
Homing success probability (24 h after release) [1]	**0.500 (a)**	**0.659 (b)**	**0.512 (a)**	**0.337 (c)**
Result of Kruskal-Wallis test on homing duration (24 h after release)	**H = 2.467, df = 3, P = 0.481**			

[1]Pairwise comparisons were performed with binomial tests and used Bonferroni P value adjustment method. Same letters indicate no significant differences.

D) Laboratory 5

	Control	Thiam. 0.11 ng/bee	Thiam. 0.33 ng/bee	Thiam. 1 ng/bee
Number of foragers released	88	81	75	78
Homing success probability (24 h after release) [1]	**0.648 (a)**	**0.642 (a)**	**0.693 (a)**	**0.500 (b)**
Result of Kruskal-Wallis test on homing duration (24 h after release)	**H= 1.763, df = 3, P = 0.623**			

[1]Pairwise comparisons were performed with binomial tests and used Bonferroni P value adjustment method. Same letters indicate no significant differences.

E) Laboratory 6

	Control	Thiam. 0.11 ng/bee	Thiam. 0.33 ng/bee	Thiam. 1 ng/bee
Number of foragers released	83	88	88	81
Homing success probability (24 h after release) [1]	**0.639 (a)**	**0.682 (ab)**	**0.580 (ac)**	**0.358 (d)**
Result of Kruskal-Wallis test on homing duration (24 h after release)	**H = 5.110, df = 3, P = 0.164**			

[1]Pairwise comparisons were performed with binomial tests and used Bonferroni P value adjustment method. Same letters indicate no significant differences.

F) Laboratory 7

	Control	Thiam. 0.11 ng/bee	Thiam. 0.33 ng/bee	Thiam. 1 ng/bee
Number of foragers released	124	122	111	111
Homing success probability (24 h after release) [1]	**0.823 (a)**	**0.820 (a)**	**0.874 (a)**	**0.568 (b)**
Result of Kruskal-Wallis test on homing duration (24 h after release)	**H = 0.713, df = 3, P = 0.870**			

[1]Pairwise comparisons were performed with binomial tests and used Bonferroni P value adjustment method. Same letters indicate no significant differences.

G) Laboratory 8

	Control	Thiam. 0.11 ng/bee	Thiam. 0.33 ng/bee	Thiam. 1 ng/bee
Number of foragers released	96	101	97	99
Homing success probability (24 h after release) [1]	**0.844 (a)**	**0.782 (a)**	**0.773 (a)**	**0.586 (b)**
Result of Kruskal-Wallis test on homing duration (24 h after release)	**H= 0.438, df = 3, P = 0.932**			

[1]Pairwise comparisons were performed with binomial tests and used Bonferroni P value adjustment method. Same letters indicate no significant differences.

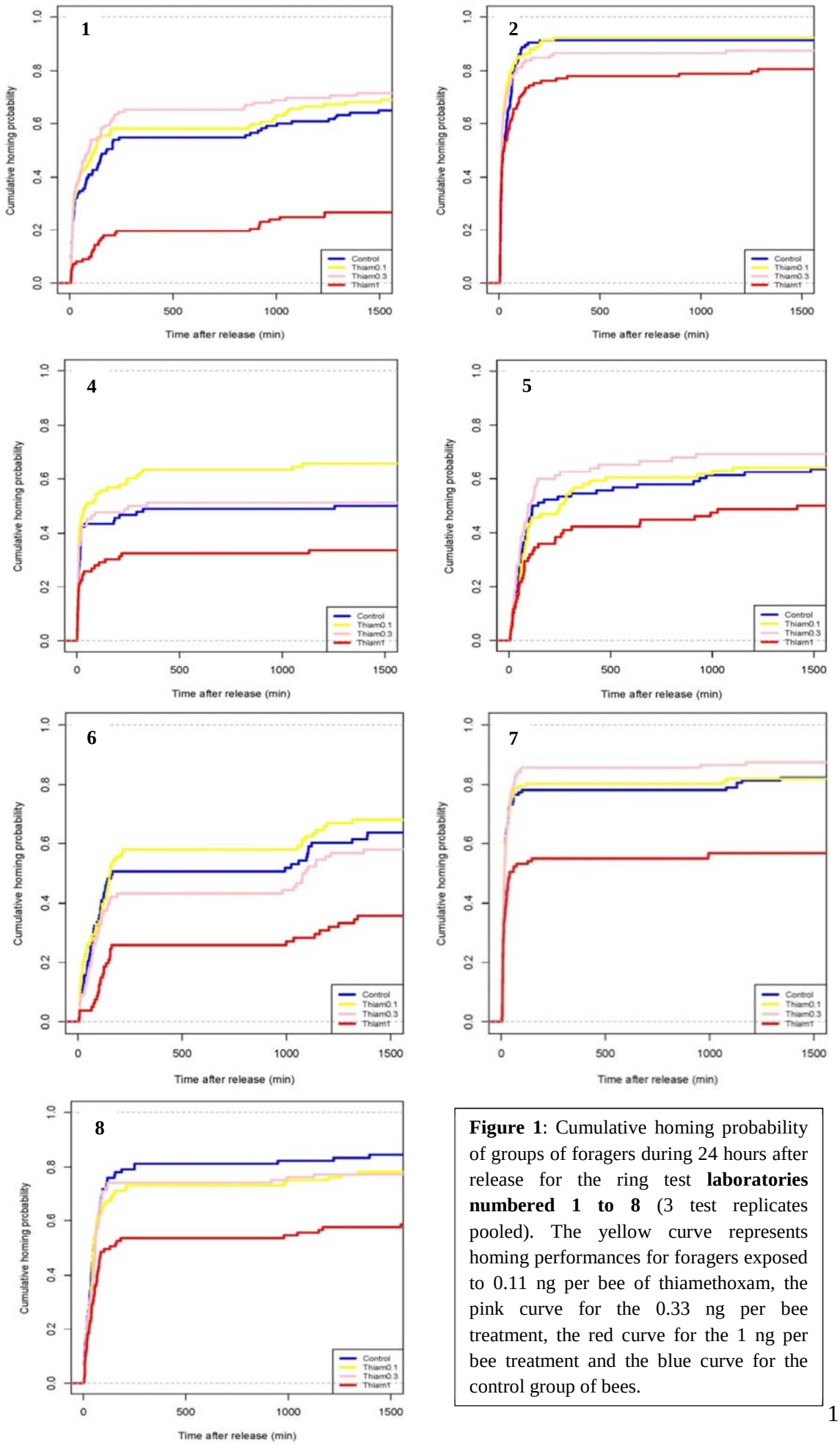

Figure 1: Cumulative homing probability of groups of foragers during 24 hours after release for the ring test **laboratories numbered 1 to 8** (3 test replicates pooled). The yellow curve represents homing performances for foragers exposed to 0.11 ng per bee of thiamethoxam, the pink curve for the 0.33 ng per bee treatment, the red curve for the 1 ng per bee treatment and the blue curve for the control group of bees.

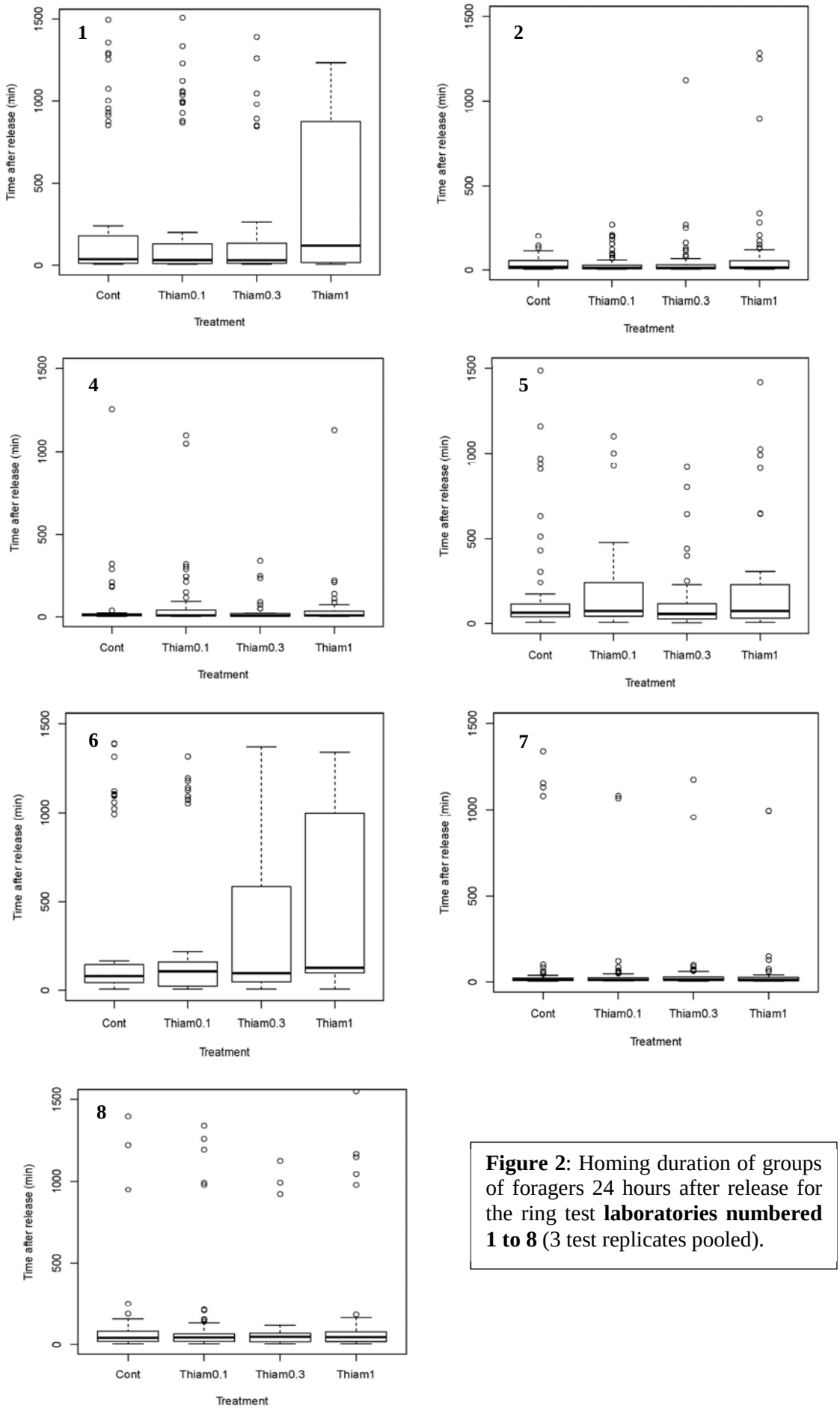

Figure 2: Homing duration of groups of foragers 24 hours after release for the ring test **laboratories numbered 1 to 8** (3 test replicates pooled).

5.3 Homing success per treatment and replicate

For each treatment, results concerning homing success 24 h-after release showed as a whole variability from one replicate test to another as well as within each laboratory (Tables 5, 6, 7).

Focusing on control bees, a majority of results ranged in homing success classes [50-60[and [90-100[(Table 5). From these results, a minimum homing success of 60% in control bees might be considered as a validity criterion for each test replicate of the method.
For two control results, lower homing flight rates were obtained (homing success class [30-40[). Four laboratories also experienced higher variability in control group from one replicate to another (Table 6).

Table 5: Results of homing successes per classes (%) for each treatment and test replicates

	Homing success classes (%)									
Treatment	**[0-10[**	**[10-20[**	**[20-30[**	**[30-40[**	**[40-50[**	**[50-60[**	**[60-70[**	**[70-80[**	**[80-90 [**	**[90-100 [**
Control	**0**	**0**	**0**	**2**	**0**	**4**	**6**	**1**	**3**	**6**
Thiamethoxam 0.11 ng/bee	0	0	0	2	0	2	3	4	**7**	4
Thiamethoxam 0.33 ng/bee	0	0	1	1	1	1	4	**6**	**6**	2
Thiamethoxam 1 ng/bee	2	4	1	2	1	**5**	1	4	0	2

A total of 22 test replicates were performed for the homing flight ring test labs 2015. Values indicated in blue correspond to the highest number of test results for the homing success class considered.

Table 6: Results of homing successes per classes (%) for each treatment and laboratories

Treatment	Laboratory	Homing probability classes (%)									
		[0-10[	[10-20[	[20-30[	[30-40[	[40-50[	[50-60[	[60-70[	[70-80[	[80-90 [	[90-100 [
Control	**1**						2			1	
	2									1	2
	3							1			
	4				1		1	1			
	5						1	2			
	6				1			1			1
	7							1			2
	8								1	1	1
Thiamethoxam 0.11 ng/bee	**1**						1	1			1
	2									1	2
	3							1			
	4				1					2	
	5						1	1	1		
	6				1					2	
	7								1	1	1
	8								2	1	
Thiamethoxam 0.33 ng/bee	**1**						1		1	1	
	2									2	1
	3					1					
	4			1				2			
	5							1	1	1	
	6				1			1	1		
	7									2	1
	8								3		
Thiamethoxam 1 ng/bee	**1**	1	1				1				
	2								2		1
	3		1								
	4		1		1		1				
	5		1				1		1		
	6	1			1			1			
	7			1		1					1
	8						2		1		

Table 7: Results of homing success probabilities per test replicate and laboratory

Laboratory	Replicate	Control	Thiam. 0.1ng/bee	Thiam. 0.3 ng/bee	Thiam. 1 ng/bee
1	**1**	0.897	0.950	0.800	0.579
	2	0.564	0.600	0.769	0.027
	3	0.513	0.514	0.588	0.189
2	**1**	0.865	0.816	0.882	0.711
	2	0.921	1	0.907	0.929
	3	0.950	0.949	0.824	0.765
3	**1**	0.641	0.675	0.421	0.154
4	**1**	0.533	0.821	0.636	0.345
	2	0.667	0.800	0.667	0.567
	3	**0.300**	**0.367**	**0.267**	**0.100**
5	**1**	0.667	0.733	0.700	0.759
	2	0.643	0.545	0.882	0.100
	3	0.600	0.621	0.679	0.517
6	**1**	**0.321**	**0.367**	**0.333**	**0.040**
	2	0.963	0.862	0.633	0.667
	3	0.643	0.828	0.786	0.345
7	**1**	0.927	0.923	0.974	0.921
	2	0.927	0.833	0.838	0.278
	3	0.619	0.707	0.806	0.486
8	**1**	0.806	0.794	0.759	0.529
	2	0.966	0.862	0.786	0.778
	3	0.778	0.711	0.775	0.500

One part of homing results obtained for control honeybees could be explained by natural mortality. Indeed we work with foragers, which are older bees. Then, variation due to natural mortality in field condition can explain a part of homing failure and results variability from one replicate to another. In addition, different factors could play an important role and explain the variability for control results. Environmental conditions like climatic conditions experienced during homing flight test are a first factor. Temperatures have especially shown to play an important role in the modulation of the homing flight results (Henry et al. 2014). Homing success increases with temperatures. Sanitary conditions of the colonies are also an important factor. For each laboratory, apiarist visits were performed before the experiments to select well-developing and healthy colonies, free from visible diseases (high *Varroa sp.* load, *Nosema sp.* or foulbrood symptoms). Experience acquired with the homing flight method can also be important. Practice and experience gain help with bees handling and experimental conditions for the test. Finally, it could also be discussed within the ring test group to prolong RFID recording period after release from 24 hours up to 48 hours for a next ring test in 2016. Indeed, for laboratories 1, 5, 6, 7 and 8, some bees have shown to return relatively later to their colony even if a large majority of the bees come back during the 24 hours period after release (Figure 2). This is especially observed for control bees. This could be so interesting to prolong homing recording for some bees that could come back later to the hive.

Climatic conditions recorded during the homing flight tests 24h-after bees' release are presented in Table 8.

Table 8: Mean climatic conditions recorded during 24 hours after bees' release for each laboratory and replicate

Laboratory	Replicate	Mean temperature (°C)	Mean hygrometry (%)	Mean rainfall (mm)
1	1	26.00	57.02	0
	2	20.76	50.62	0
	3	23.56	52.14	0
2	**1**	18.63	62.92	0
	2	21.67	61.52	1
	3	25.31	54.06	0
3	**1**	16.38	54.68	0
4	**1**	20.18	75.54	5.7
	2	22.84	69.44	5
	3	23.20	68,42	2.5
5	**1**	25.05	62.58	0
	2	26.45	59.55	0
	3	24.99	58.66	0
6	**1**	**14.40**	67.28	0.2
	2	18.32	60.68	0.7
	3	18.44	67.28	0.1
7	**1**	20.46	70.08	1
	2	19.32	77.60	1.5
	3	16.97	80.96	**8**
8	**1**	20.32	62.30	0
	2	19.40	59.28	0
	3	22.48	52.28	0

The laboratory 6 experienced cool temperatures for the first test replicate that can explain the low homing flight results (Table 7). The laboratory 4 also experienced low homing flight results for the third test replicate (Table 7), but climatic conditions are not a possible explanation as they were relatively favorable during experiments of the laboratory 4 (Table 8). In parallel, this laboratory didn't experienced problem of mortality before the bees' releases (Table 3).

The laboratory 5 experienced problems with bee conditions in laboratory as a whole. Bees' weakness appeared during starvation period and they had to be fed ad-libitum with sucrose solution during post-exposure starvation period to avoid mortality for replicates 1 and 3. High mortalities were recorded for replicate 2 conducted according to the ring test protocol 2015 (Table 2). Bee's physiology, possible scarce food supplies in the environment at the time of the experiment combined with high temperatures conditions could explain this problem. However, feeding the bees during post-exposure starvation period changed exposure conditions and no more differences in homing results were found between control and bees exposed to the highest dose of 1 ng per bee of thiamethoxam for replicates 1 and 3 (Table 7).

Possible methodological adjustments will be discussed with the ring test group for a next ring test in 2016.
For one test replicate of the laboratories 2 and 7, no differences in homing success of bees exposed to the highest dose and control bees were also recorded (Table 7).

5.4 Result of the analyses of test item solutions

To determine the acceptance criteria, we considered the mean dose value of all real doses analysed more or less the analytical incertitude of 35 %. For the expected nominal dose of 1 ng per bee, two high outliers values of more than 2 ng per bee were determined after analytical analyses (Table 9). These two values were excluded for the determination of the acceptance criteria. Then for the nominal dose of 1 ng per bee, mean dose value of the ring test group calculated was 1.132 ng per bee with the acceptance interval [0.736-1.528]. For the nominal dose of 0.33 ng per bee, mean dose value of the ring test group calculated was 0.391 ng per bee with the acceptance interval [0.254-0.528]. For the lowest nominal dose of 0.11 ng per bee, mean dose value of the ring test group calculated was 0.115 ng per bee with the acceptance interval [0.075-0.155] (Table 9).

Table 9: Results of the analytical analyses on test sucrose solution for each test replicate and laboratory.

Lab	Replicate	Nominal dose : 1 ng/bee 0.736 < D < 1.528	Nominal dose : 0.33 ng/bee 0.254 < D < 0.528	Nominal dose : 0.11 ng/bee 0.075 < D < 0.155
1	1	1.688	0.462	0.084
	2	2.160	0.532	0.134
	3	1.388	0.538	0.110
2	1	0.986	0.312	0.096
	2	1.024	0.334	0.090
	3	1.164	0.272	0.070
3	1	1.200	0.394	0.112
4	1	1.116	0.390	0.112
	2	1.072	0.298	0.114
	3	1.076	0.460	0.164
5	1	1.366	0.486	0.152
	2	1.142	0.328	0.100
	3	0.972	0.308	0.096
6	1	0.884	0.346	0.098
	2	0.662	0.318	0.106
	3	1.028	0.388	0.122
7	1	2.216	0.452	0.144
	2	1.384	0.522	0.114
	3	1.360	0.434	0.122
8	1	0.902	0.318	0.130
	2	1.044	0.332	0.130
	3	1.188	0.372	0.124

Most of the measured doses were in the respective acceptance intervals determined for the highest, the intermediate and the lowest tested doses.
For the highest tested dose, one outlier of 2.16 ng per bee (replicate 2) and one measured dose of 1.688 ng per bee (replicate 1), out of the acceptance interval, were measured for the laboratory 1. The outlier value didn't induce higher mortality before release (Table 2) and all the bees consumed the sucrose solution administered. But it could have an impact on homing results as low homing success rate was obtained for bees exposed to this treatment in test replicate 2 compared to replicates 1 and 3 (Table 7). For the group of bees exposed to the measured dose of 1.688 ng per bee in replicate 1, no mortality was recorded after exposure (Table 1) and there was no clear effect on homing success compared to the results of the third test replicate for this laboratory.
For the laboratory 7, an outlier of 2.216 ng per bee was measured in test replicate 1. As for the laboratory 1, this did not induce higher mortality before release (Table 1). However, homing success was not impacted and did not differ from homing success of control bees (Table 7).
On the opposite, a lower dose of 0.662 ng per bee out of the acceptance interval was measured for the second test replicate of the laboratory 6. For this test replicate, homing success results didn't differ between bees exposed to this highest dose and bees exposed to the real intermediate dose of 0.318 ng per bee (Tables 7 and 9).
For the intermediate and lowest tested doses, some measured values were out of the respective acceptance interval, but this had not impact on homing success results compared to the homing success of control bees (Tables 7 and 9).

5.5 Additional experiments

5.5.1 Pre-tests performed with acetone solvent

Pre-tests were internally performed to assess possible acetone effects on homing flight when used as a test control. Two tests with two replicates per test could be performed in June (Test 1, laboratory ITSAP/ACTA) and July (Test 2, laboratory INRA Le Magneraud) 2015. All types of foragers carrying pellets of pollen or not were considered for the tests. Replicates for test 1 were conducted with the same colony whereas each replicate of test 2 were performed with a different colony. We tested 3 different volumes of acetone including the highest acceptable volume for a homing test (0.1 % v/v, 0.5 % v/v and 1 % v/v in the 30 % w/v sucrose solution) and we compared to a water control (1% v/v in the 30 % w/v sucrose solution). Then, bees were labelled with a RFID microchip according to treatment tested and underwent same exposure conditions than for the ring tests experiments 2015 (see material and method section). For test 1, bees were released at one site, at 1 km (+/- 100 m) of the experimental hive whereas for test 2, bees were released in equal group at three sites equally spaced along a 1 km-radius around the experimental colony.

Considering the results for the 2 tests, the different tested acetone volume would not show any effect on homing flight performances as a whole. For the two tests especially, bees exposed to the highest volume of acetone (1% v/v) returned to the hive in the same proportions than bees exposed to water in feeding solution (exact binomial tests, $P > 0.05$; Table 10, A and B). Homing duration estimated 24 hours after release didn't significantly differ between groups of bees (Kruskal-wallis tests, $P > 0.05$; Table 10, A and B). Details of statistical analyses for homing success and homing duration for the tests 1 and 2 are presented in **Appendix 5.**

Table 10: Homing flight test results for acetone experiments (A and B)
Results of the two replicates pooled together for the Test 1 (A) and 2 (B) are presented

A)Test 1 (ITSAP/ACTA)	**Control**	**Acetone 0.1% v/v**	**Acetone 0.5% v/v**	**Acetone 1% v/v**
Number of foragers released	60	60	60	59
Homing success probability (24 h after release) [1]	**0.483 (a)**	**0.500 (a)**	**0.550 (a)**	**0.458 (a)**
Result of Kruskal-Wallis test on homing duration (24 h after release)	**H = 0.105, df = 3, P = 0.991**			

[1]Pairwise comparisons were performed with binomial tests and used Bonferroni P value adjustment method. Same letters indicate no significant differences.

B) Test 2 (INRA Le Magneraud)	**Control**	**Acetone 0.1% v/v**	**Acetone 0.5% v/v**	**Acetone 1% v/v**
Number of foragers released	74	74	77	76
Homing success probability (24 h after release) [1]	**0.757 (a)**	**0.716 (a)**	**0.597 (bc)**	**0.645 (ac)**
Result of Kruskal-Wallis test on homing duration (24 h after release)	**H = 3.833, df = 3, P = 0.280**			

[1]Pairwise comparisons were performed with binomial tests and used Bonferroni P value adjustment method. Same letters indicate no significant differences.

5.5.2 Pre-tests with a possible methodological alternative to the Phacelia field

One possible identified limiting point of the methodology was the plantation of the Phacelia field. Phacelia field is important as it ensures that the bees used for the experiment are familiar with their environment. However, it imposes a fixed and limited experimental period (Phacelia blooming) with the possibility to have other attractive blooming crops at the same time or unfavorable climatic conditions. This can reduce the number of Phacelia foragers captured for the experiment. For this ring test 2015, 3 laboratories experienced problems with the Phacelia field. Then, in parallel to this ring test, a possible alternative method to the sowing of a Phacelia plot was internally pre-tested to collect bees experienced with their environment.

Principle of the alternative method

Foragers' bees carrying pellets of pollen or not were captured at the hive entrance in the morning during effective foraging period. They were placed in small box per group of 100 to 200 bees. A total of 500 to 600 bees were captured. The bees were transported to a release site at 1 km (+/- 100 m) away from the experimental colonies. Hydrophobic powder (pink fluorescent pigments – T series, COLOREY SAS, France) was added in each box with bees and quantity brought was in accordance to the number of bees captured in the box. Boxes were gently shaked in order to color the bees. Then, bees were released. At the hive entrance of the experimental colony, colored bees that returned to the hive were collected within a maximum of 2 hours. Bees captured had so at least one homing experience to the hive from the release site. A minimum of 125 bees (one test replicate) to 200 powdered bees (the other test replicates) returning to the hive were captured for the experiments. Then, bees were labelled with a RFID microchip according to treatment tested and underwent same exposure conditions than for the ring tests experiments 2015 (see material and method section).

Two tests were performed. Test 1 (laboratory ITSAP/ACTA) aimed to compare homing flight performances of "powdered" foragers and of all types of foragers that have different experiences of the environment. All types of foragers were released in equal group at six sites equally spaced along a 1 km-radius around the experimental colony whereas "powdered" bees were released at the same site they were released the first time after powdering, at 1 km (+/- 100 m) of the experimental hive. Test 2 (laboratory INRA Le Magneraud) aimed to conduct a homing test with "powdered" bees and the test item thiamethoxam. For test 2, "powdered" bees were all released at the same site they were released the first time after powdering, at 1 km (+/- 100 m) of the experimental hive.
Two replicates were performed for each test in mid-August (Test 1) and September (Test 2) 2015. Each replicate of test 1 and 2 were performed with a different colony.

First results and discussion

Test 1: Comparison of homing flight performances of "powdered" foragers *vs* control bees (all types of foragers; laboratory ITSAP/ACTA)

Powdered bees returned to the hive in significantly higher proportions than control bees (all types of foragers bees) collected at the hive entrance (exact binomial test, $P < 0.05$; Table 11). Homing duration 24 hours after release significantly differed between groups of bees. Powdered bees group came back quicker to the hive than "all types of bees" group (Mann-Whitney test, U=2140; P=0.037, Table 11) Details of statistical analyses for homing success probabilities and homing duration of the Test 1 are presented in **Appendix 6**.

Table 11: Homing flight test results for control (all types of foragers) vs "powdered" bees (Test 1-ITSAP/ACTA). Results of the two replicates pooled together are presented

	Control "all types of bees	**Powdered bees**
Number of foragers released	116	117
Homing success probability (24 h after release) [1]	**0.457 (a)**	**0.564 (b)**
Results of Mann-Whitney test on homing duration (24 h after release)	**U= 2140, P = 0.037**	

[1]Pairwise comparisons were performed with binomial tests. Different letters indicate significant differences.

Test 2: Homing flight of "powdered" bees with test item thiamethoxam (laboratory INRA Le Magneraud)

Powdered bees exposed to the highest dose of thiamethoxam at 1 ng per bee returned to the hive in significantly lower proportions than the bees exposed to control or the two other tested treatments ($P < 0.0001$; Table 12). However for this test, bees exposed to thiamethoxam at 0.11 and 0.33 ng per bee also returned to the hive in significantly lower proportions than the bees exposed to control treatment ($P< 0.001$; Table 12). Homing duration estimated 24 hours after release didn't significantly differ between groups of bees. Details of statistical analyses for homing probability and homing duration for the test 2 are presented in **Appendix 6**.

Table 12: Homing flight results for "powdered" bees with test item thiamethoxam (Test 2-INRA Le Magneraud). Results of the two replicates pooled together are presented

	Control	**Thiam. 0.11 ng/bee**	**Thiam. 0.33 ng/bee**	**Thiam. 1 ng/bee**
Number of foragers released	**74**	**77**	**72**	**73**
Homing success probability (24 h after release) [1]	**0.784 (a)**	**0.597(b)**	**0.597(b)**	**0.329 (c)**
Result of Kruskal-Wallis test on homing duration (24 h after release)	**H = 2.184, df = 3, P = 0.535**			

[1]Pairwise comparisons were performed with binomial tests and used Bonferroni P value adjustment method. Different letters indicate significant differences.

Only two replicates were performed for each pre-test and these pre-tests could be performed relatively late in the experimental season (mid-August and September). But from the first results obtained, the "powder" method could offer an interesting and easier alternative to the use of a Phacelia field for the homing test performance. We especially avoid limiting experimental period imposed by the Phacelia blooming period.

CONCLUSION

Eleven European laboratories participated to the first international homing flight ring test 2015 with two laboratories working together. Seven laboratories could completely conduct the test and one laboratory conducted partially the test from June to August, according to the ring test protocol.

The seven laboratories that completely conduct the test (3 replicates) found an effect of the highest sublethal tested dose of 1 ng of thiamethoxam per bee on the homing flight of foragers. Bees exposed to the highest dose returned to the hive in significantly lower proportions than control bees. All the laboratories also found the same NOED of 0.33 ng per bee on the homing flight which was the test endpoint. Then, these results point out the sensitivity and the suitability of the test method to detect possible effects of low doses of a test chemical on the homing flight behaviour, important component of the foraging activity.

Considering control bees, results have also shown variability in homing success from one replicates to another and from one laboratory to another. This variability could be explained by different factors like climatic conditions or experience with the method. However a majority of homing success probabilities in control bees ranged from 60 to more than 90 %. Then a minimum homing success of 60% for control bees might be set as a validity criterion for each test replicate of the method.

One laboratory experienced problems with bees' conditions during laboratory phase. Bees' weakness appeared during starvation periods and they had to be fed ad-libitum with sucrose solution during post-exposure starvation period to avoid mortality. High mortalities were recorded for one replicate conducted according to the ring test protocol 2015. Bee's physiology, possible scarce food supplies in the environment at the time of the experiment combined with high temperatures conditions could explain this problem. However, feeding the bees during post-exposure starvation period changed exposure conditions and no more homing differences were found between control and bees exposed to 1 ng per bee of thiamethoxam for the test replicates with ad libitum sucrose solution supply after exposure.

Finally, three participating laboratories experienced problems with the Phacelia field and forager's capture. In parallel to this ring test, we internally tested a possible alternative method to the plantation of a Phacelia plot. The method tested using a powder to recognize and collect bees experienced with their environment could offer an interesting and easier alternative to the use of a Phacelia field for the homing test performance. Other pre-tests with acetone solvent were internally conducted and as a whole would not show any effect on homing flight performances. Additional experiments will be conducted in 2016 to complete these results.

The validity criterion, possible methodological adjustments and alternative method to Phacelia field will be discussed at a feedback meeting with the ring test laboratories in January 2016. Then, another ring test will be conducted in 2016. Next year, one laboratory more will join the ring test group (Agroscope, Switzerland).

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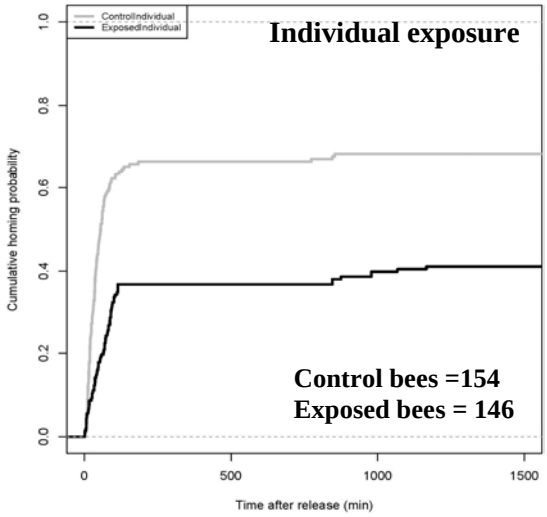
APPENDIX 1

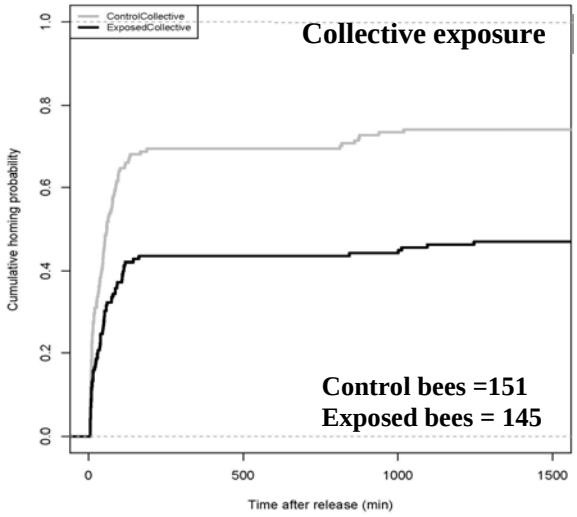
Homing flight results 2014

In 2014, homing success probabilities for bees exposed to 1 ng per bee of thiamethoxam were compared to control bees (acetone 0.1 % v/v in 30% w/v sucrose solution). Individual and collective mode of exposure, per group of 10 foragers' bees, were tested. Five test replicates were performed, each one with a different colony

1) Homing success probabilities of control (grey) and treated (black) bees during 24 hours after release

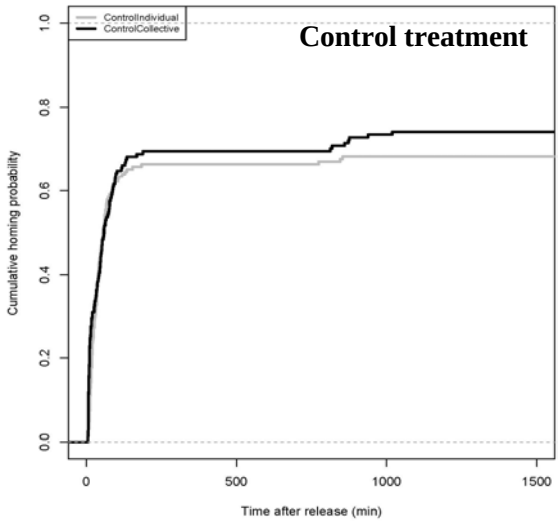


Binomial test 24-h after release **P< 0.001**

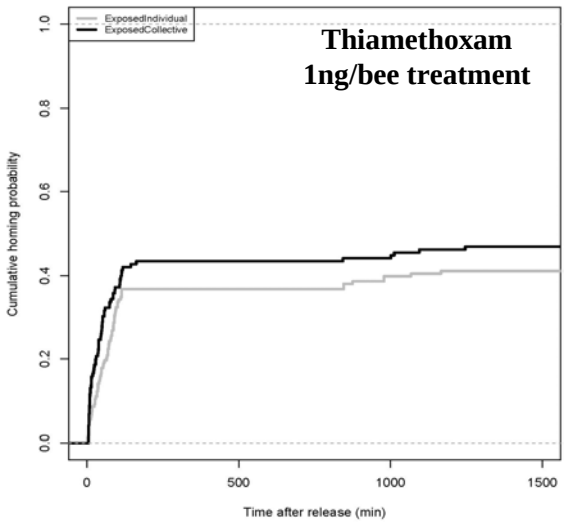


Binomial test 24-h after release **P< 0.001**

2) Homing success probabilities of bees individually (grey) and collectively exposed (black) during 24 hours after release



Binomial test 24-h after release **P=0.934**



Binomial test 24-h after release **P=0.933**

APPENDIX 2

Certificate of Analysis

Dr. Ehrenstorfer

Reference Materials for Residue Analysis

Product Identification

17453000 Thiamethoxam

CA 4H-1,3,5-Oxadiazin-4-imine, 3-[(2-chloro-5-thiazolyl)methyl]tetrahydro-5-methyl-N-nitro-

IUPAC 3-(2-Chloro-1,3-thiazol-5-ylmethyl)-5-methyl-1,3,5-oxadiazinan-4-ylidene(nitro)amine

Formula C8H10ClN5O3S

Mol.Weight 291.71

CAS No. 153719-23-4

Expiry Date 19.11.2017

Lot Number 31119

Store at 20 °C ±4 °C

Please note: The expiry date is valid under recommended storage conditions only.

Toxicological Data

R Code 22-53

S Code 60/61

LD50 (Rats female/male in mg/kg) 1563

Physical Data

Phase	crystalline solid	Vapour pressure 6.6E-6 mPa at 25 °C
Color	colourless	Solubility in water 4.1 g/l at 25 °C
Melt.Range	138.9 °C	Boiling Range (lit.)

Analytical Data

Detection:	HPLC/DAD	Method Details:
Column:	ReproSil 100 C18 5µ 250x3	Acetonitrile:H2O 4:1
Inj.-Vol.:	10.00 µl	
Flow:	1.0 ml/min	
Ret.-Time:	**1.04 min.**	

Identity: UV, RT

Comment Purity was determined by external standard method.

Water Content 0.0 % Determined by Karl-Fischer Titration

Det. Purity 99.0 % Tolerance/Uncertainty +/- 0.5 %

The uncertainty/tolerance of this standard is calculated in accordance with the EURACHEM/CITAC Guide - Quantifying Uncertainty in Analytical Measurement - Second Edition. The uncertainty given is the expanded combined uncertainty and represents an estimated standard deviation equal to the positive square root of the total variance of the uncertainty of components. The expanded uncertainty is U which is Uc(y)*K, where K is the coverage factor at the 95% confidence level (K=2). The expanded uncertainty is based on the combination of uncertainties associated with each individual operation involved in the preparation of this product.

Certified on 15.01.2014

by A. Storr

The Laboratory Labor Dr. Ehrenstorfer-Schäfers is accreditated by DAkkS as indicated by the Accreditation Number D-RM-14174-01 has shown competence based on ISO Guide 34:2009 with relevant parts of DIN EN ISO/IEC 17025:2005 for production of certified reference materials in form of organic pure substances and in form of single and multi-component solutions organic pure substances.

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Phone +49 821 906080 · Fax +49 821 9060888 · info@analytical-standards.com

Data File D:\CHEM32\1\DATA\201401KW02\174530001.D
Sample Name: 40109AL 31119

Thiamethoxam

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Sample Name     : 40109AL 31119                Location : Vial 56
Acq. Operator   : DAD1_Admin                       Inj :  1
Acq. Instrument : Instrument 1             Inj Volume : 10 µl
Method          : D:\CHEM32\1\METHODS\41K.M
Last changed    : 05.12.2013 10:04:25  by DAD1_Admin
Acetonitrile : Water 4:1
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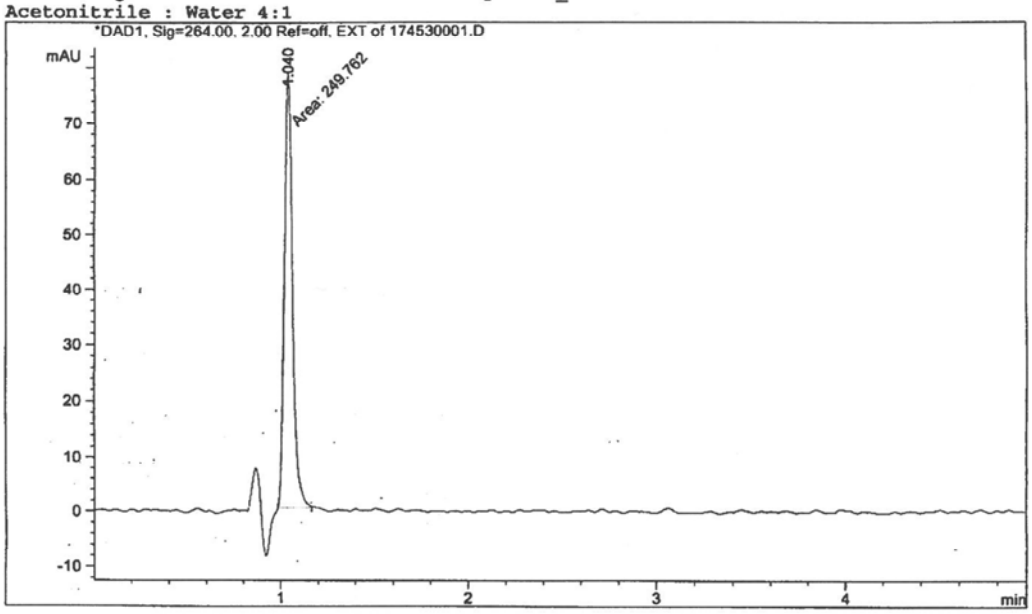


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======================================================================
                        Area Percent Report
======================================================================

Sorted By             :      Retention Time
Multiplier            :      1.0000
Dilution              :      1.0000
Use Multiplier & Dilution Factor with ISTDs

Signal 1: DAD1, Sig=264.00, 2.00 Ref=off, EXT
 Signal has been modified after loading from rawdata file!

Peak RetTime Sig Type    Area      Height     Area
  #   [min]            [mAU*s]     [mAU]       %
----|-------|---|----|----------|----------|--------|
   1   1.040  1  MM T  249.76204   78.99778 100.0000

Totals :               249.76204   78.99778

======================================================================
                        *** End of Report ***
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APPENDIX 3
Preparation of the test item and test feeding solutions

- ⇨ 20 µl of sucrose solution (30 % w/v) per bee containing 0.1% of acetone is considered
- ⇨ Test item doses: 0.11, 0.33 and 1 ng per bee

1- Preparation of the stock solution (S)

**1 ng test item in 0.02 µl acetone => 50 ng/µl or 50 µg/ml*

Preparation of a one hundred time more concentrated « S » :

50 x 100 = 5 000 µg/ml or 5 mg/ml

To prepare « S » => 10 mg of thiamethoxam is weighed and 2 ml of acetone is added

2- Preparation of a 1ng per bee test solution (S1)

Dilution 1/100 : solution « S1 » at 50 µg/ml

10 ml as a final acetone volume is considered.

Preparation :

Ci xVi = Cf xVf => 5000 µg/ml x Vi = 50 µg/ml x 10

Vi = 0,1 ml => 100 µl of S is sampled and 9.9 ml of acetone is added

3- Preparation of a 0.33 ng per bee test solution (S2)

Dilution 1/3 : solution S2 at 16.667 µg/ml

Preparation : 1 ml of S1 is sampled and 2 ml of acetone is added

4- Preparation of a 0.11 ng per bee test solution (S3)

Dilution 1/3 : solution S3 at 5.556 µg/ml

Preparation : 1 ml of S2 is sampled and 2 ml of acetone is added

5- Test feeding solutions

General preparation : 15 g of sugar in 50 ml of demineralised water (30 % w/v)

Four samples of 10 ml of this sucrose solution are prepared for the 3 tested and control treatments.

For the test feeding solutions, it is prepareed in 10 ml of sucrose solution :

Treatment	Test solution sample in µl	Sucrose solution (30% w/v) in ml
Control (acetone)	10 µl acetone	10
Thiamethoxam 1 ng	10 µl S1	10
Thiamethoxam 0.33 ng	10 µl S2	10
Thiamethoxam 0.11 ng	10 µl S3	10

APPENDIX 4

Homing flight ring test 2015 : statistical analysis performed on homing rates and homing duration 24 hours after bees release

Laboratory 1

A) Homing rate : Pairwise comparisons with binomial tests (P<0.05) and with Bonferroni P value adjustment method

Thiam. 1 ng/bee vs control Exact binomial test data: c (sum(retour_Thiam1), sum(!retour_Thiam1)) number of successes = 30, number of trials = 112, **p-value < 2.2e-16** alternative hypothesis: true probability of success is less than 0.6608696 **95 percent confidence interval:** **0.0000000 0.3453207** sample estimates: probability of success 0.2678571 **p-value adjustment with Bonferroni method : 6.6e-16**	***Thiam. 0.11 ng/bee vs control*** Exact binomial test data: c(sum(retour_Thiam0.1), sum(!retour_Thiam0.1)) number of successes = 81, number of trials = 117, **p-value = 0.7917** alternative hypothesis: true probability of success is less than 0.6608696 **95 percent confidence interval:** **0.0000000 0.7624471** sample estimates: probability of success 0.6923077 **p-value adjustment with Bonferroni method : 1**
Thiam. 1 ng/bee vs Thiam 0.11 ng/bee Exact binomial test data: c(sum(retour_Thiam1), sum(!retour_Thiam1)) number of successes = 30, number of trials = 112, **p-value < 2.2e-16** alternative hypothesis: true probability of success is less than 0.6923077 **95 percent confidence interval:** **0.0000000 0.3453207** sample estimates: probability of success 0.2678571 **p-value adjustment with Bonferroni method : 6.6e-16**	***Thiam. 0.33 ng/bee vs control*** Exact binomial test data: c(sum(retour_Thiam0.3), sum(!retour_Thiam0.3)) number of successes = 82, number of trials = 113, **p-value = 0.942** alternative hypothesis: true probability of success is less than 0.6608696 **95 percent confidence interval:** **0.0000000 0.7940011** sample estimates: probability of success 0.7256637 **p-value adjustment with Bonferroni method : 1**
Thiam. 1 ng/bee vs Thiam 0.33 ng/bee Exact binomial test data: c(sum(retour_Thiam1), sum(!retour_Thiam1)) number of successes = 30, number of trials = 112, **p-value < 2.2e-16** alternative hypothesis: true probability of success is less than 0.7256637 **95 percent confidence interval:** **0.0000000 0.3453207** sample estimates: probability of success 0.2678571 **p-value adjustment with Bonferroni method : 6.6e-16**	***Thiam. 0.33 ng/bee vs Thiam. 0.11 ng/bee*** Exact binomial test data: c(sum(retour_Thiam0.3), sum(!retour_Thiam0.3)) number of successes = 82, number of trials = 113, **p-value = 0.807** alternative hypothesis: true probability of success is less than 0.6923077 **95 percent confidence interval:** **0.0000000 0.7940011** sample estimates: probability of success 0.7256637

B) Homing duration : Kruskal-Wallis test

Kruskal-Wallis rank sum test

data: ab$rfid_release_min by ab$Treat

Kruskal-Wallis chi-squared = 4.9734, df = 3, **p-value = 0.1738**

Laboratory 2

A) Homing rate : Pairwise comparisons with binomial tests (P<0.05) and with Bonferroni P value adjustment method

Thiam. 1 ng/bee vs control Exact binomial test data: c(sum(retour_Thiam1), sum(!retour_Thiam1)) number of successes = 92, number of trials = 114, **p-value = 0.0003094** alternative hypothesis: true probability of success is less than 0.9130435 **95 percent confidence interval:** **0.0000000 0.8655857** sample estimates: probability of success 0.8070175 **p-value adjustment with Bonferroni method : 0.0009282**	***Thiam. 0.11 ng/bee vs control*** Exact binomial test data: c(sum(retour_Thiam0.1), sum(!retour_Thiam0.1)) number of successes = 107, number of trials = 116, **p-value = 0.6874** alternative hypothesis: true probability of success is less than 0.9130435 **95 percent confidence interval:** **0.0000000 0.9589292** sample estimates: probability of success 0.9224138 **p-value adjustment with Bonferroni method:1**
Thiam. 1 ng/bee vs Thiam 0.11 ng/bee Exact binomial test data: c(sum(retour_Thiam1), sum(!retour_Thiam1)) number of successes = 92, number of trials = 114, **p-value = 6.043e-05** alternative hypothesis: true probability of success is less than 0.9224138 **95 percent confidence interval:** **0.0000000 0.8655857** sample estimates: probability of success 0.8070175 **p-value adjustment with Bonferroni method : 0.0001812**	***Thiam. 0.33 ng/bee vs control*** Exact binomial test data: c(sum(retour_Thiam0.3), sum(!retour_Thiam0.3)) number of successes = 97, number of trials = 111, **p-value = 0.1016** alternative hypothesis: true probability of success is less than 0.9130435 **95 percent confidence interval:** **0.0000000 0.9221115** sample estimates: probability of success 0.8738739 **p-value adjustment with Bonferroni method:0.2032**
Thiam. 1 ng/bee vs Thiam 0.33 ng/bee Exact binomial test data: c(sum(retour_Thiam1), sum(!retour_Thiam1)) number of successes = 92, number of trials = 114, **p-value = 0.0272** alternative hypothesis: true probability of success is less than 0.8738739 **95 percent confidence interval:** **0.0000000 0.8655857** sample estimates: probability of success 0.8070175 **p-value adjustment with Bonferroni method : 0.0816000**	***Thiam. 0.33 ng/bee vs Thiam. 0.11 ng/bee*** Exact binomial test data: c(sum(retour_Thiam0.3), sum(!retour_Thiam0.3)) number of successes = 97, number of trials = 111, **p-value = 0.04869** alternative hypothesis: true probability of success is less than 0.9224138 **95 percent confidence interval:** **0.0000000 0.9221115** sample estimates: probability of success 0.8738739

B) Homing duration : Kruskal-Wallis test

Kruskal-Wallis rank sum test
data: ab$rfid_release_min by ab$Treat
Kruskal-Wallis chi-squared = 7.0231, df = 3, **p-value = 0.07116**

Laboratory 4

A) Homing rate : Pairwise comparisons with binomial tests (P<0.05) and with Bonferroni P value adjustment method

Thiam. 1 ng/bee vs control Exact binomial test data: c(sum(retour_Thiam1), sum(!retour_Thiam1)) number of successes = 30, number of trials = 89, **p-value = 0.001393** alternative hypothesis: true probability of success is less than 0.5 **95 percent confidence interval:** **0.0000000 0.4283718** sample estimates: probability of success 0.3370787 **p-value adjustment with Bonferroni method : 4.1790e-03**	***Thiam. 0.11 ng/bee vs control*** Exact binomial test data: c(sum(retour_control), sum(!retour_control)) number of successes = 45, number of trials = 90, **p-value = 0.001342** alternative hypothesis: true probability of success is less than 0.6590909 **95 percent confidence interval:** **0.0000000 0.5912285** sample estimates: probability of success 0.5 **p-value adjustment with Bonferroni method : 0.002684**
Thiam. 1 ng/bee vs Thiam 0.11 ng/bee Exact binomial test data: c(sum(retour_Thiam1), sum(!retour_Thiam1)) number of successes = 30, number of trials = 89, **p-value = 5.937e-10** alternative hypothesis: true probability of success is less than 0.6590909 **95 percent confidence interval:** **0.0000000 0.4283718** sample estimates: probability of success 0.3370787 **p-value adjustment with Bonferroni method : 1.7811e-09**	***Thiam. 0.33 ng/bee vs control*** Exact binomial test data: c(sum(retour_control), sum(!retour_control)) number of successes = 45, number of trials = 90, **p-value = 0.4496** alternative hypothesis: true probability of success is less than 0.5121951 **95 percent confidence interval:** **0.0000000 0.5912285** sample estimates: probability of success 0.5 **p-value adjustment with Bonferroni method : 0.899200**
Thiam. 1 ng/bee vs Thiam 0.33 ng/bee Exact binomial test data: c(sum(retour_Thiam1), sum(!retour_Thiam1)) number of successes = 30, number of trials = 89, **p-value = 0.0006421** alternative hypothesis: true probability of success is less than 0.5121951 **95 percent confidence interval:** **0.0000000 0.4283718** sample estimates: probability of success 0.3370787 **p-value adjustment with Bonferroni method : 1.9263e-03**	***Thiam. 0.33 ng/bee vs Thiam. 0.11 ng/bee*** Exact binomial test data: c(sum(retour_Thiam0.3), sum(!retour_Thiam0.3)) number of successes = 42, number of trials = 82, **p-value = 0.004228** alternative hypothesis: true probability of success is less than 0.6590909 **95 percent confidence interval:** **0.0000000 0.6075711** sample estimates: probability of success 0.5121951

B) Homing duration : Kruskal-Wallis test

Kruskal-Wallis rank sum test
data: ab$rfid_release_min by ab$Treat
Kruskal-Wallis chi-squared = 2.467, df = 3, **p-value = 0.4813**

Laboratory 5

A) Homing rate : Pairwise comparisons with binomial tests (P<0.05) and with Bonferroni P value adjustment method

Thiam. 1 ng/bee vs control
Exact binomial test
data: c(sum(retour_Thiam1), sum(!retour_Thiam1))
number of successes = 39, number of trials = 78, **p-value = 0.005176**
alternative hypothesis: true probability of success is less than 0.6477273
95 percent confidence interval:
0.0000000 0.5982586
sample estimates:
probability of success
0.5
p-value adjustment with Bonferroni method: 0.0155280

Thiam. 0.11 ng/bee vs control
Exact binomial test
data: c(sum(retour_Thiam0.1), sum(!retour_Thiam0.1))
number of successes = 52, number of trials = 81, **p-value = 0.4986**
alternative hypothesis: true probability of success is less than 0.6477273
95 percent confidence interval:
0.0000000 0.7306439
sample estimates:
probability of success
0.6419753
p-value adjustment with Bonferroni method : 0.9972

Thiam. 1 ng/bee vs Thiam 0.11 ng/bee
Exact binomial test
data: c(sum(retour_Thiam1), sum(!retour_Thiam1))
number of successes = 39, number of trials = 78, **p-value = 0.007044**
alternative hypothesis: true probability of success is less than 0.6419753
95 percent confidence interval:
0.0000000 0.5982586
sample estimates:
probability of success
0.5
p-value adjustment with Bonferroni method : 0.0211320

Thiam. 0.33 ng/bee vs control
Exact binomial test
data: c(sum(retour_Thiam0.3), sum(!retour_Thiam0.3))
number of successes = 52, number of trials = 75, **p-value = 0.828**
alternative hypothesis: true probability of success is less than 0.6477273
95 percent confidence interval:
0.0000000 0.7804866
sample estimates:
probability of success
0.6933333
p-value adjustment with Bonferroni method : 1.0000

Thiam. 1 ng/bee vs Thiam 0.33 ng/bee
Exact binomial test
data: c(sum(retour_Thiam1), sum(!retour_Thiam1))
number of successes = 39, number of trials = 78, **p-value = 0.0002793**
alternative hypothesis: true probability of success is less than 0.6933333
95 percent confidence interval:
0.0000000 0.5982586
sample estimates:
probability of success
0.5
p-value adjustment with Bonferroni method : 0.0008379

Thiam. 0.33 ng/bee vs Thiam. 0.11 ng/bee
Exact binomial test
data: c(sum(retour_Thiam0.3), sum(!retour_Thiam0.3))
number of successes = 52, number of trials = 75, **p-value = 0.853**
alternative hypothesis: true probability of success is less than 0.6419753
95 percent confidence interval:
0.0000000 0.7804866
sample estimates:
probability of success
0.6933333

B) Homing duration : Kruskal-Wallis test

Kruskal-Wallis rank sum test
data: ab$rfid_release_min by ab$Treat
Kruskal-Wallis chi-squared = 1.7629, df = 3, **p-value = 0.623**

Laboratory 6

A) Homing rate : Pairwise comparisons with binomial tests (P<0.05) and with Bonferroni P value adjustment method

Thiam. 1 ng/bee vs control
Exact binomial test
data: c(sum(retour_Thiam1), sum(!retour_Thiam1))
number of successes = 29, number of trials = 81, **p-value = 2.727e-07**
alternative hypothesis: true probability of success is less than 0.6385542
95 percent confidence interval:
0.0000000 0.4547216
sample estimates:
probability of success
0.3580247
p-value adjustment with Bonferroni method : 8.181e-07

Thiam. 0.11 ng/bee vs control
Exact binomial test
data: c(sum(retour_Thiam0.1), sum(!retour_Thiam0.1))
number of successes = 60, number of trials = 88, **p-value = 0.8302**
alternative hypothesis: true probability of success is less than 0.6385542
95 percent confidence interval:
0.0000000 0.7635164
sample estimates:
probability of success
0.6818182
p-value adjustment with Bonferroni method : 1

Thiam. 1 ng/bee vs Thiam 0.11 ng/bee
Exact binomial test
data: c(sum(retour_Thiam1), sum(!retour_Thiam1))
number of successes = 29, number of trials = 81, **p-value = 2.244e-09**
alternative hypothesis: true probability of success is less than 0.6818182
95 percent confidence interval:
0.0000000 0.4547216
sample estimates:
probability of success
0.3580247
p-value adjustment with Bonferroni method : 6.732e-09

Thiam. 0.33 ng/bee vs control
Exact binomial test
data: c(sum(retour_Thiam0.3), sum(!retour_Thiam0.3))
number of successes = 51, number of trials = 88, **p-value = 0.149**
alternative hypothesis: true probability of success is less than 0.6385542
95 percent confidence interval:
0.00000 0.66869
sample estimates:
probability of success
0.5795455
p-value adjustment with Bonferroni method : 0.298

Thiam. 1 ng/bee vs Thiam 0.33 ng/bee
Exact binomial test
data: c(sum(retour_Thiam1), sum(!retour_Thiam1))
number of successes = 29, number of trials = 81, **p-value = 4.83e-05**
alternative hypothesis: true probability of success is less than 0.5795455
95 percent confidence interval:
0.0000000 0.4547216
sample estimates:
probability of success
0.3580247
p-value adjustment with Bonferroni method : 1.449e-04

Thiam. 0.33 ng/bee vs Thiam. 0.11 ng/bee
Exact binomial test
data: c(sum(retour_Thiam0.3), sum(!retour_Thiam0.3))
number of successes = 51, number of trials = 88, **p-value = 0.02781**
alternative hypothesis: true probability of success is less than 0.6818182
95 percent confidence interval:
0.00000 0.66869
sample estimates:
probability of success
0.5795455

B) Homing duration : Kruskal-wallis test

Kruskal-Wallis rank sum test
data: ab$rfid_release_min by ab$Treat
Kruskal-Wallis chi-squared = 5.1101, df = 3, **p-value = 0.1639**

Laboratory 7

A) Homing rate : Pairwise comparisons with binomial tests (P<0.05) and with Bonferroni P value adjustment method

Thiam. 1 ng/bee vs control
Exact binomial test
data: c(sum(retour_Thiam1), sum(!retour_Thiam1))
number of successes = 63, number of trials = 111, **p-value = 4.002e-10**
alternative hypothesis: true probability of success is less than 0.8225806
95 percent confidence interval:
0.0000000 0.6473144
sample estimates:
probability of success
0.5675676
p-value adjustment with Bonferroni method : 1.2006e-09

Thiam. 1 ng/bee vs Thiam 0.11 ng/bee
Exact binomial test
data: c(sum(retour_Thiam1), sum(!retour_Thiam1))
number of successes = 63, number of trials = 111, **p-value = 7.04e-10**
alternative hypothesis: true probability of success is less than 0.8196721
95 percent confidence interval:
0.0000000 0.6473144
sample estimates:
probability of success
0.5675676
p-value adjustment with Bonferroni method : 2.1120e-09

Thiam. 1 ng/bee vs Thiam 0.33 ng/bee
Exact binomial test
data: c(sum(retour_Thiam1), sum(!retour_Thiam1))
number of successes = 63, number of trials = 111, **p-value = 1.24e-15**
alternative hypothesis: true probability of success is less than 0.8738739
95 percent confidence interval:
0.0000000 0.6473144
sample estimates:
probability of success
0.5675676
p-value adjustment with Bonferroni method : 3.7200e-15

Thiam. 0.11 ng/bee vs control
Exact binomial test
data: c(sum(retour_Thiam0.1), sum(!retour_Thiam0.1))
number of successes = 100, number of trials = 122, **p-value = 0.5035**
alternative hypothesis: true probability of success is less than 0.8225806
95 percent confidence interval:
0.0000000 0.8746476
sample estimates:
probability of success
0.8196721
p-value adjustment with Bonferroni method : 1

Thiam. 0.33 ng/bee vs control
Exact binomial test
data: c(sum(retour_Thiam0.3), sum(!retour_Thiam0.3))
number of successes = 97, number of trials = 111, **p-value = 0.9434**
alternative hypothesis: true probability of success is less than 0.8225806
95 percent confidence interval:
0.0000000 0.9221115
sample estimates:
probability of success
0.8738739
p-value adjustment with Bonferroni method : 1

Thiam. 0.33 ng/bee vs Thiam. 0.11 ng/bee
Exact binomial test
data: c(sum(retour_Thiam0.3), sum(!retour_Thiam0.3))
number of successes = 97, number of trials = 111, **p-value = 0.9516**
alternative hypothesis: true probability of success is less than 0.8196721
95 percent confidence interval:
0.0000000 0.9221115
sample estimates:
probability of success
0.8738739

B) Homing duration : Kruskal-Wallis test

Kruskal-Wallis rank sum test
data: ab$rfid_release_min by ab$Treat
Kruskal-Wallis chi-squared = 0.7134, df = 3, **p-value = 0.87**

Laboratory 8

A) Homing rate : Pairwise comparisons with binomial tests (P<0.05) and with Bonferroni P value adjustment method

Thiam. 1 ng/bee vs control
Exact binomial test
data: c(sum(retour_Thiam1), sum(!retour_Thiam1))
number of successes = 58, number of trials = 99, **p-value = 7.375e-10**
alternative hypothesis: true probability of success is less than 0.84375
95 percent confidence interval:
0.0000000 0.6695117
sample estimates:
probability of success
0.5858586
p-value adjustment with Bonferroni method : 2.2125e-09

Thiam. 0.11 ng/bee vs control
Exact binomial test
data: c(sum(retour_Thiam0.1), sum(!retour_Thiam0.1))
number of successes = 79, number of trials = 101, **p-value = 0.06304**
alternative hypothesis: true probability of success is less than 0.84375
95 percent confidence interval:
0.0000000 0.8476876
sample estimates:
probability of success
0.7821782
p-value adjustment with Bonferroni method : 0.12608

Thiam. 1 ng/bee vs Thiam 0.11 ng/bee
Exact binomial test
data: c(sum(retour_Thiam1), sum(!retour_Thiam1))
number of successes = 58, number of trials = 99, **p-value = 8.974e-06**
alternative hypothesis: true probability of success is less than 0.7821782
95 percent confidence interval:
0.0000000 0.6695117
sample estimates:
probability of success
0.5858586
p-value adjustment with Bonferroni method : 2.6922e-05

Thiam. 0.33 ng/bee vs control
Exact binomial test
data: c(sum(retour_Thiam0.3), sum(!retour_Thiam0.3))
number of successes = 75, number of trials = 97, **p-value = 0.04289**
alternative hypothesis: true probability of success is less than 0.84375
95 percent confidence interval:
0.0000000 0.8411785
sample estimates:
probability of success
0.7731959
p-value adjustment with Bonferroni method : 0.08578

Thiam. 1 ng/bee vs Thiam 0.33 ng/bee
Exact binomial test
data: c(sum(retour_Thiam1), sum(!retour_Thiam1))
number of successes = 58, number of trials = 99, **p-value = 2.484e-05**
alternative hypothesis: true probability of success is less than 0.7731959
95 percent confidence interval:
0.0000000 0.6695117
sample estimates:
probability of success
0.5858586
p-value adjustment with Bonferroni method : 7.4520e-05

Thiam. 0.33 ng/bee vs Thiam. 0.11 ng/bee
Exact binomial test
data: c(sum(retour_Thiam0.3), sum(!retour_Thiam0.3))
number of successes = 75, number of trials = 97, **p-value = 0.4545**
alternative hypothesis: true probability of success is less than 0.7821782
95 percent confidence interval:
0.0000000 0.8411785
sample estimates:
probability of success
0.7731959

B) Homing duration : Kruskal-Wallis test

Kruskal-Wallis rank sum test
data: ab$rfid_release_min by ab$Treat
Kruskal-Wallis chi-squared = 0.4384, df = 3, **p-value = 0.9322**

APPENDIX 5
Pre-tests performed with acetone solvent:statistical analysis performed on homing rates and homing duration 24 hours after bee release

TEST 1

A) Homing rate : Pairwise comparisons with binomial tests (P<0.05) with Bonferroni P value adjustment method

Acetone 1% (v/v) vs Water control 1% (v/v) Exact binomial test data: c(sum(retour_Acet1), sum(!retour_Acet1)) number of successes = 27, number of trials = 59, **p-value = 0.3962** alternative hypothesis: true probability of success is less than 0.4833333 **95 percent confidence interval:** **0.0000000 0.5724816** sample estimates: probability of success 0.4576271 **p-value adjustment with Bonferroni method : 1**	***Acetone 0.1% (v/v) vs Water control 1% (v/v)*** Exact binomial test data: c(sum(retour_Acet0.1), sum(!retour_Acet0.1)) number of successes = 30, number of trials = 60, **p-value = 0.6511** alternative hypothesis: true probability of success is less than 0.4833333 **95 percent confidence interval:** **0.000000 0.612589** sample estimates: probability of success 0.5 **p-value adjustment with Bonferroni method : 1**
Acetone 1% (v/v) vs Acetone 0.1% (v/v) Exact binomial test data: c(sum(retour_Acet1), sum(!retour_Acet1)) number of successes = 27, number of trials = 59, **p-value = 0.3015** alternative hypothesis: true probability of success is less than 0.5 **95 percent confidence interval:** **0.0000000 0.5724816** sample estimates: probability of success 0.4576271 **p-value adjustment with Bonferroni method: 0.90450**	***Acetone 0.5% (v/v) vs Water control 1% (v/v)*** Exact binomial test data: c(sum(retour_Acet0.5), sum(!retour_Acet0.5)) number of successes = 33, number of trials = 60, **p-value = 0.8775** alternative hypothesis: true probability of success is less than 0.4833333 **95 percent confidence interval:** **0.0000000 0.6601575** sample estimates: probability of success 0.55 **p-value adjustment with Bonferroni method : 1**
Acetone 1% (v/v) vs Acetone 0.5% (v/v) Exact binomial test data: c(sum(retour_Acet1), sum(!retour_Acet1)) number of successes = 27, number of trials = 59, **p-value = 0.09789** alternative hypothesis: true probability of success is less than 0.55 **95 percent confidence interval:** **0.0000000 0.5724816** sample estimates: probability of success 0.4576271 **p-value adjustment with Bonferroni method: 0.29367**	***Acetone 0.5% (v/v) vs Acetone 0.1% (v/v)*** Exact binomial test data: c(sum(retour_Acet0.5), sum(!retour_Acet0.5)) number of successes = 33, number of trials = 60, **p-value = 0.8169** alternative hypothesis: true probability of success is less than 0.5 **95 percent confidence interval:** **0.0000000 0.6601575** sample estimates: probability of success 0.55

B) Homing duration : Kruskal-Wallis test

Kruskal-Wallis rank sum test
data: ab$rfid_release_min by ab$Treat
Kruskal-Wallis chi-squared = 0.1054, df = 3, **p-value = 0.9912**

TEST 2

A) Homing rate : Pairwise comparisons with binomial tests (P<0.05) with Bonferroni P value adjustment method

Acetone 1% (v/v) vs Water control 1% (v/v) Exact binomial test data: c(sum(retour_Acet1), sum(!retour_Acet1)) number of successes = 49, number of trials = 76, **p-value = 0.01891** alternative hypothesis: true probability of success is less than 0.7567568 **95 percent confidence interval:** **0.0000000 0.7360247** sample estimates: probability of success 0.6447368 **p-value adjustment with Bonferroni method : 0.05673**	***Acetone 0.1% (v/v) vs Water control 1% (v/v)*** Exact binomial test data: c(sum(retour_Acet0.1), sum(!retour_Acet0.1)) number of successes = 53, number of trials = 74, **p-value = 0.2452** alternative hypothesis: true probability of success is less than 0.7567568 **95 percent confidence interval:** **0.0000000 0.8013065** sample estimates: probability of success 0.7162162 **p-value adjustment with Bonferroni method : 0.490400**
Acetone 1% (v/v) vs Acetone 0.1% (v/v) Exact binomial test data: c(sum(retour_Acet1), sum(!retour_Acet1)) number of successes = 49, number of trials = 76, **p-value = 0.1063** alternative hypothesis: true probability of success is less than 0.7162162 **95 percent confidence interval:** **0.0000000 0.7360247** sample estimates: probability of success 0.6447368 **p-value adjustment with Bonferroni method : 0.31890**	***Acetone 0.5% (v/v) vs Water control 1% (v/v)*** Exact binomial test data: c(sum(retour_Acet0.5), sum(!retour_Acet0.5)) number of successes = 46, number of trials = 77, **p-value = 0.001449** alternative hypothesis: true probability of success is less than 0.7567568 **95 percent confidence interval:** **0.0000000 0.6916755** sample estimates: probability of success 0.5974026 **p-value adjustment with Bonferroni method: 0.002898**
Acetone 1% (v/v) vs Acetone 0.5% (v/v) Exact binomial test data: c(sum(retour_Acet1), sum(!retour_Acet1)) number of successes = 49, number of trials = 76, **p-value = 0.8309** alternative hypothesis: true probability of success is less than 0.5974026 **95 percent confidence interval:** **0.0000000 0.7360247** sample estimates: probability of success 0.6447368 **p-value adjustment with Bonferroni method : 1**	***Acetone 0.5% (v/v) vs Acetone 0.1% (v/v)*** Exact binomial test data: c(sum(retour_Acet0.5), sum(!retour_Acet0.5)) number of successes = 46, number of trials = 77, **p-value = 0.01653** alternative hypothesis: true probability of success is less than 0.7162162 **95 percent confidence interval:** **0.0000000 0.6916755** sample estimates: probability of success 0.5974026

B) Homing duration : Kruskal-Wallis test

Kruskal-Wallis rank sum test
data: ab$rfid_release_min by ab$Treat
Kruskal-Wallis chi-squared = 3.8328, df = 3, **p-value = 0.2801**

APPENDIX 6

Pre-tests with a possible methodological alternative to the Phacelia field: statistical analysis performed on homing rates and homing duration 24 hours after bee release

TEST 1 ***:*** Comparison of homing flight performances of "powdered" foragers *vs* all types of foragers (laboratory ITSAP/ACTA)

A) Homing rate : Comparisons with binomial tests (P<0.05)

Exact binomial test
data: c(sum(retour_control), sum(!retour_control))
number of successes = 53, number of trials = 116, **p-value = 0.01303**
alternative hypothesis: true probability of success is less than 0.5641026
95 percent confidence interval:
0.0000000 0.5374431
sample estimates:
probability of success
0.4568966

B) Homing duration : Mann-Whitney test

Wilcoxon rank sum test with continuity correction
data: ab$rfid_release_min by ab$Treat
W = 2140, **p-value = 0.03681**

<u>TEST 2</u>: Homing flight of "powdered" bees with test item thiamethoxam (laboratory INRA Le Magneraud)

A) Homing rate : Pairwise comparisons with binomial tests (P<0.05) with Bonferroni P value adjustment method

Thiam. 1 ng/bee vs control Exact binomial test data: c(sum(retour_Thiam1), sum(!retour_Thiam1)) number of successes = 24, number of trials = 73, **p-value < 2.2e-16** alternative hypothesis: true probability of success is less than 0.7837838 **95 percent confidence interval:** **0.0000000 0.4301925** sample estimates: probability of success 0.3287671 **p-value adjustment with Bonferroni method : 6.6e-16**	***Thiam. 0.11 ng/bee vs control*** Exact binomial test data: c(sum(retour_Thiam0.1), sum(!retour_Thiam0.1)) number of successes = 46, number of trials = 77, **p-value = 0.0001705** alternative hypothesis: true probability of success is less than 0.7837838 **95 percent confidence interval:** **0.0000000 0.6916755** sample estimates: probability of success 0.5974026 **p-value adjustment with Bonferroni method : 0.0003410**
Thiam. 1 ng/bee vs Thiam 0.11 ng/bee Exact binomial test data: c(sum(retour_Thiam1), sum(!retour_Thiam1)) number of successes = 24, number of trials = 73, **p-value = 3.213e-06** alternative hypothesis: true probability of success is less than 0.5974026 **95 percent confidence interval:** **0.0000000 0.4301925** sample estimates: probability of success 0.3287671 **p-value adjustment with Bonferroni method : 9.639e-06**	***Thiam. 0.33 ng/bee vs control*** Exact binomial test data: c(sum(retour_Thiam0.3), sum(!retour_Thiam0.3)) number of successes = 43, number of trials = 72, **p-value = 0.0002699** alternative hypothesis: true probability of success is less than 0.7837838 **95 percent confidence interval:** **0.0000000 0.6947521** sample estimates: probability of success 0.5972222 **p-value adjustment with Bonferroni method : 0.0005398**
Thiam. 1 ng/bee vs Thiam 0.33 ng/bee Exact binomial test data: c(sum(retour_Thiam1), sum(!retour_Thiam1)) number of successes = 24, number of trials = 73, p-value = 3.261e-06 alternative hypothesis: true probability of success is less than 0.5972222 95 percent confidence interval: 0.0000000 0.4301925 sample estimates: probability of success 0.3287671 **p-value adjustment with Bonferroni method : 9.783e-06**	***Thiam. 0.33 ng/bee vs Thiam. 0.11 ng/bee*** Exact binomial test data: c(sum(retour_Thiam0.3), sum(!retour_Thiam0.3)) number of successes = 43, number of trials = 72, **p-value = 0.5435** alternative hypothesis: true probability of success is less than 0.5974026 **95 percent confidence interval:** **0.0000000 0.6947521** sample estimates: probability of success 0.5972222

B) Homing duration : Kruskal-Wallis test

Kruskal-Wallis rank sum test
data: ab$rfid_release_min by ab$Treat
Kruskal-Wallis chi-squared = 2.1836, df = 3, **p-value = 0.5352**

Part II - Final report:
Results of the international ring test 2016 and 2017 Validation of the Homing flight test in honeybee (Apis mellifera L.) after single exposure to sublethal doses of test chemical

Results of the international ring test 2016 and 2017
Final report

Validation of the Homing flight test in honeybee (Apis mellifera L.) after single exposure to sublethal doses of test chemical

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31 March 2020

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1-INTRODUCTION

According to the Regulation (EC) No 1107/2009 (Annex II point 3.8.3), an active substance or a formulated plant protection product, shall only be approved if it is carefully evaluated following an appropriate risk assessment. Among several factors, this includes acute and chronic effects on colony survival and colony development, considering effects on honeybee larvae and **honeybee behaviour**. For the latter, there exists no standardized method to evaluate the effect of sublethal doses on foraging honeybees. Sublethal effects in individual worker bees may have the potential to affect functions at the colony level and, or colony survival (Henry et al. 2012, 2015, Woodcock et al. 2017). Recent revision of plant protection products' risk assessment on bees recommended the use of a homing flight test to study the effect of sublethal doses of plant protection products on this trait of interest (EFSA, 2013).

The homing test proposes to assess effects of a single, oral exposure to sublethal doses of a chemical (technical grade active substance or a formulation) on the homing performance of forager bees. Thereby, feeding solutions are administered under controlled conditions and subsequently foragers are released in order to mimic field realistic homing conditions.

The method project was adopted by the OECD Working Group of National Coordinators of the Test Guidelines Programme (WNT) and is integrated in the work plan of OECD since 2016.

Having demonstrated the transferability of the test method in 2014 by French laboratories (ITSAP/ACTA and INRA Le Magneraud), the homing test was presented to a broader audience at the international ICPPR symposium in Ghent (Belgium). Then, an OECD ring test group has been founded consisting of eleven European laboratories (including Germany, United Kingdom, Switzerland, Italy, and France). After a training phase, a first ring test trial session was performed in 2015. These results were presented in a previous report ("Summary of results of the First international ring test 2015 for the standardisation of a homing flight test design", December 11, 2015). Subsequently, the ring test trials continued and corresponding results obtained during 2016 and 2017 are presented in the actual report here. From 2016 on, an additional laboratory from Switzerland has joined the working group. The tests' endpoint is the determination of a No-Observed-Effect-Dose (NOED) on the homing success of foragers released at a distance of 1 km (+/-100 m) away from the experimental colony. This distance is within the range that foragers routinely cover during normal foraging flights (Steffan-Dewenter and Kuhn, 2003). Moreover, the proposed ring test aims to establish a validity criterion of the studies regarding the minimum- and acceptable homing-success-rate of untreated control bees.
The active substance thiamethoxam was used as a reference item in this ring test, since several studies have demonstrated that thiamethoxam can negatively affect the homing ability of foragers (Henry et al. 2012, 2015). For each trial we tested three sublethal doses of the active substance (according to a geometric progression with a ratio of 3): 0.11 ng, 0.33 ng and 1 ng per bee. Similarly, a control solution (acetone 0.1 % in a 30 % w/v sucrose solution) was included. All labs used technical grade thiamethoxam originating from the same batch number (purity = 99%). For each test run, bees were exposed collectively (in 10 bees-cages) to one of the four feeding solutions.

From 2016 on, an alternative method to that of the Phacelia approach was used to ensure that foragers were familiar with their environment. The approach with the Phacelia field allowed to collect bees with specific bright blue pollen loads, but similarly presented some fundamental limitations (fixed and limited experimental period during Phacelia blooming, impact of poor climatic conditions on plant growth etc…). As a result of this, three out of ten laboratories couldn't perform the test in 2015. The alternative method is based on the use of a colored powder, which allows to recognize bees at the hive entrance that were released at 1 km (+/-100 m) away from the experimental colony. Using

this method, it can be ensured that foragers have at least one prior knowledge of the pathway back to the colony.
Methodological improvements were continuously achieved based on experimental observations. For example, such knowledge was used to improve the conditions that facilitate the maintainance of the foragers during the laboratory phase.

Homing performance was measured (for 24 hours) by monitoring free-ranging foragers with radio-frequency identification (RFID) tagging technology. For each treatment-group, both, homing success rate and its corresponding duration were calculated from the automatically saved data. For the interpretation of obtained results, the variability and potential causing factors were discussed.

2-INFORMATION ON THE RING TEST GROUP

Totally twelve laboratories participated in the ring test of 2016 and 2017. Since two laboratories worked together for the ring test (CREA-API and Biotecnologie BT S.r.l), 11 laboratories were counted to perform the test. Participants represented a wide range of stakeholder groups, including governmental institutions, contract laboratories and technical bee institutes.

Laboratory	**Responsible person(s)**
ITSAP-Institut de l'Abeille, **France** ***Project leader*** ***Organiser of the ring test***	Julie Fourrier (ITSAP)
INRA Le Magneraud, **France** ***Organiser of the ring test***	Pierrick Aupinel Dominique Fortini Colombe Chevallereau
Innovative Environmental Services (IES) Ltd, **Switzerland**	Bettina Saar Stefan Kimmel
-CRA-API, **Italy** -Biotecnologie BT S.r.l, **Italy**	Piotr Medrzycki Irene Guerra Monica Colli Simone Venturi
Agroscope, **Switzerland**	Lukas Jeker Michael Eyer
The Fera (Science) Ltd, **United Kingdom**	Selwyn Wilkins Emma Wright
ibacon, Institut für Biologische Analytik und Consulting Gmbh, **Germany**	Thomas Bing Stephan Schmitzer
Eurofins Agroscience Services Ecotox GmbH, **Germany**	Marco Kleinhenz Annette Kling
BioChem agrar GmbH, **Germany**	Markus Barth Melanie Hänsel
LAVES Institute for Apidology Celle, **Germany**	Martina Janke Dorothee Lueken
TESTAPI, **France**	Hervé Giffard Claire Molitor Olivier Mamet

Organisation and coordination of this work was supported by grants from the French Ministry of Agriculture (FranceAgriMer) and Lune de Miel® Foundation.

3-RING TEST SCHEDULE (2016 and 2017)

Start of experimental phase	May 2016/2017
End of experimental phase	September 2016/2017
Evaluation of results and	July to December 2016/2017
Results presentation to the ring test group	January 2017/2018

4-MATERIAL AND METHODS

4.1 Honeybees

Source of the colonies, treatments and health status: Chemical treatments (anti-varroa…) have been completed at least four weeks before the start of the experiment. Queen-right (queens with known history and not older than 2 years) and healthy colonies (as far as possible disease-free) were used for the experiments.

Hives characteristics: Each test hive was equipped with 10 to 12-frames. To ensure that bees did correctly circulate through RFID readers entrances and to prevent trophallaxis at the bottom of the hive between bees within the hive and those outside, laboratories employed different floor systems according to summer temperatures and conditions: **1)** hives with a full floor, **2)** a system consisting of two mesh floors fixed together and separated by few centimetres, **3)** two half mesh floors placed in a staggered configuration. One to two supers could be added on top of the hive to increase the hives' volume and to ensure good thermoregulation during summer climatic conditions.

Preparation of the colonies: The colonies used for the ring test were homogenous in terms of colony strength, food storage, amount of brood and experimental preparation. For hives with ten frames configuration they comprised four to seven brood combs of all stages (eggs, uncapped larvae and pupae) and for hives with twelve frames configuration they contained six to eight brood combs. All hives further contained two to three food combs and at least one empty frame. Good colony activity was checked by monitoring the foraging activity at the hive entrance. A colony inspection (routine apiarist visit) was performed for each experimental colony one to four days before the test start to prepare colony and to verify health status.

Installation of the colonies: All the colonies used for the test were installed on the experimental site, at least one week before the start of the test, to allow acclimatisation and familiarisation to the environment by the honeybees. The colonies were separated spatially by a few meters (≥ 3 meters) and placed in a staggered configuration to maximally avoid drift of labelled bees between the colonies.

4.2 RFID device

RFID (Radio Frequency Identification) device: The RFID technology (Streit *et al*. 2003; Decourtye *et al*. 2011) allows detection each time an RFID tagged bee passed through the reader (working distance of 3 mm). The principle depends on the emission of a radio signal from the reader which is received by the tag on the bee's thorax. The tag is not equipped with a power source (passive

function) and it obtains its operating power from the reading process to emit a unique identification code. Each tag had a unique identification and it is possible for the readers to automatically recognize a virtually unlimited number of tagged individual insects. Another strength of this method is that the signal can cross physical barriers such as propolis, wood, plastic or dust.

The tags used were 13.56 MHz frequency; Microsensys GmbH, Erfurt, Germany (2.0 x 1.7 x 0.5 mm).They weighed no more than 3 mg, equivalent to approximately 3 % of the weight of a worker bee. RFID tags were glued on the thorax of the bees.

The RFID system used was MAJA system (Microsensys GmbH, Erfurt, Germany). It comprised of one Host (small computer with a Windows system) that recorded data of all forager passing's on a SD card. Four readers were placed at the entrance of the hive (parallel arrangement). Each reader spanned a tunnel of 14 × 21.5 mm (7 mm high) acting as an entrance to the colony. Readers were installed at the hive entrance thanks to an interface (in plastic or wood) between hive and readers (= mask). Then, the bees were able to enter the hive through the 4 possible entrances formed by the readers (**Figure 1**, **Appendix 1**).

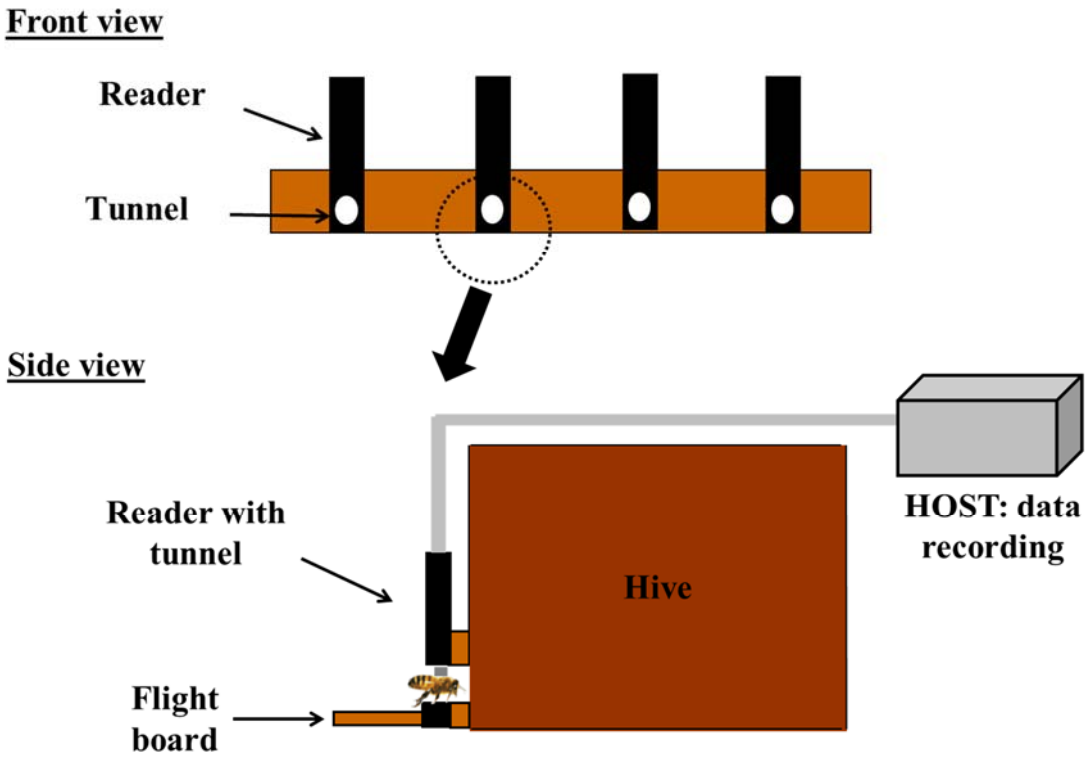


Figure 1: Picture of the RFID device

The tag's identification code (Unique identifications, or UIDs) and the exact time of the event (date, hour, minute and second) were recorded with the MAJA Host capture software. RFID data were collected by connecting the MAJA Host with a PC laptop equipped with Microsoft Mobile Device Center software. The date and time (hour, minute and second) settings for the MAJA Host and PC laptop were synchronized before data collection. To maintain continues recording, power supply was required, via either battery or electricity.

Reading rates: The acceptance for the reading was that **at least 95% of the crossing of bees should be recorded**. To ensure that this was possible, a validation test was performed – before the system was fitted to the hive – by simulating honeybees crossing with tags glued onto small plastic or wooden sticks.

Protocol to control the performance of the RFID system and RFID-reading rates 2016 and 2017 of the ring test group are presented in **Appendix 2**.

Fitting RFID equipment to the colonies: The first experimental hive was equipped with the RFID device at least **four days before the test**. For the other colonies tested, a blank platform which mimicked the RFID system was placed at the hive entrance to allow the forager bees to familiarise themselves with the entrance style prior to fitting the RFID readers for the experiment.

Tag Batches: Pre-numbered 'Tag Kits' were used to tag the bees. Each kit contained RFID Tags which had previously been read and identified - using a Pen reader to identify the UIDs of the tags in a particular kit. This information was stored in an excel spreadsheet. The kits were then allocated to a particular treatment group. This allowed the UIDs and hence the bees and kits to be tracked. Three to four batches of 10 to 15 tags (bees) were prepared per each test run and treatment.

4.3 Test item

Technical grade neonicotinoid active ingredient (a.i.) thiamethoxam
Supplier: Laboratories Dr. Ehrenstorfer-Schäfers, Augsburg
CAS number: 153719-23-4
Purity: 99.0%

All participating labs used technical grade thiamethoxam with the same batch number. Certificate of analysis 2016 and 2017 are proposed in **Appendix 3**.

4.4 Test design

Number of treatments:	1 control group and 3 test item groups
Number of bees labelled and exposed per treatment and run:	minimum of 30 bees ➔ 3 cages of 10 tested bees respectively for 30 bees tested (the cage is the experimental unit)
Number of test runs:	3, each one with a different colony

4.5 Preparation of the test item and test feeding solutions

The preparation of the test item and test feeding solutions is presented in **Appendix 4**.
Test item solutions: A stock solutions of the test item were prepared. These could be prepared in advance and stored in the refrigerator at 4 °C ± 4 °C for up to 5 days before the start of the test. Acetone was used as the solvent. An untreated control using acetone solvent was prepared (purity ≥ 98%). The final volume of the test or control solutions in the sucrose feeding solution was 0.1% (v/v). It was shown in 2015 and 2016 that up to a volume of 1% in the sucrose solution acetone had no significant effect on homing success of bees compared to bees receiving only water (**Appendix 5**).

Test feeding solutions: The test item and control solutions (acetone 0.1 % v/v) were administered in a sucrose feeding solution containing 30% (w/v, sucrose in demineralised water). In proportion, this corresponded to 30 g of sucrose in 100 ml of demineralised water.

Test feeding solutions could be prepared and stored at 4 °C ± 4 °C up to 1 day before the test.

Fresh test item or control solutions and sucrose solutions were prepared for each test run, and were stored in a deep freeze at –20°C ± 2°C after the test for the concentration and dose of the tested chemical to be analytically determined. To do so, the 3 treated test feeding solutions (≥ 5 ml of solution per sample) for the 3 test runs conducted were forwarded to ITSAP/ACTA laboratory before being sent to the French food safety agency (ANSES, Sophia Antipolis) for analytical analysis.

4.6 Test cages

All laboratories used ventilated cages of an appropriate size for the number of the foragers captured. They were designed so that the bees could be observed (either having transparent panels or being completely transparent). Cages could be opened easily to allow insertion and release of the bees.

4.7 Homing flight test procedure

Capture and preparation of "coloured" foragers
The test day in the morning and when the bees were actively foraging, returning foragers either with or without pollen were captured at the hive entrance. Different methods could be used to collect foragers:

> ***1) One by one with entomological clamps***
> The collected (up to 300 individuals per group) bees were placed in boxes (e.g. plastic food trays of 600 to 2000 cm^3 with for example 11 x 15 x 12 cm height). Bees were introduced in each box through a hole in the lid, which could be covered to prevent escape.
>
> ***2) Collectively with an insect aspirator or other suitable system***
> The collected bees were collected with an insect aspirator or other system in containers (e.g. plastic bottles of 1000 cm^3 or boxes). Containers (bottles or boxes) were weighed empty and then weighed with the bees captured using a field precision balance (e.g. max 500 g, precision 0.1 g). The weight of bees was converted into the number of bees. To do so, **a group of 20 foragers of the experimental colony** were weighed to estimate a mean weight per bee.

A minimum of 600 bees were captured. Hydrophobic Powder (pink fluorescent pigments – T series, COLOREY SAS, France) was added in each box containing captured bees with a proportion of **0.5 mg per bee in 2016** and **0.3 mg per bee in 2017**. A proportion of 0.3 mg per bee was sufficient to color the bees adequately. A preliminary acute toxicity study performed with 4 participating laboratories in 2016 showed that the pink hydrophobic powder alone or in combination with the tested doses of the test item did not lead to adverse effect on survival, sensitivity and natural behaviour of foragers compared to non-coloured bees exposed or not to the tested doses of thiamethoxam (**Appendix 6**). Boxes/bottles were gently shaken in order to color the bees evenly.

Before or after being marked with the colored powder, collected foragers were transported to a release site located at 1 km (+/- 100 m) away from the experimental colonies. Boxes/bottles were placed on a flat surface and opened to allow the bees to exit. If necessary, the bees were emptied out.

Recapture of the "coloured" foragers at the hive entrance
Recapturing of foragers: colored bees returning to the hive were collected (on the flight board) up to a maximum of 2 hours following release (**Appendix 1**). Thus, bees captured had at least one homing experience to the hive from the release site. Bees were grouped into cages (up to 40 bees per cage) with food *ad libitum*. **Candy (**e.g. Apifonda®) **was used**. If necessary, water could also be provided once the bees were captured.

Number of foragers captured: A minimum of 140 foragers must be captured to obtain at least 30 foragers per one of the four groups.

Labelling and exposure in the laboratory

Feeding, starvation and labelling phase:

Captured foragers were transferred to the laboratory (holding a constant temperature of 23 ± 3°C during the entire experimental phase). Foragers were first provided with food *ad libitum* (candy: e.g. Apifonda®) to synchronize their dietary state. The length was half of an hour in 2016 and was increased in 2017 to one hour (Henry et al. 2012). During this feeding period, cages were kept in dark conditions (e.g. half opened isolated box with a wet towel to avoid dehydration).Water could also be provided for the bees during this period.

After this period, the test started and the bees underwent a starvation phase of **90 to 120 mins duration.** During all this starvation phase, the bees were transferred one by one from the cages to a holding cage where a foam plunger allowed them to be immobilised without damage and labelled with an RFID tag (e.g. queen marking device, **Appendix 1**). The tag was glued on the thorax of the foragers using glue such as "dental cement" (e.g. Temposil®, Coltene). The glue is non-corrosive and dries very quickly (less than 2 minutes). A pre-test performed in 2016 by Lab 3 confirmed that the glue was suitable for the test system as bees coming back to the hive retained their tag (**Appendix 7**).

During the labelling phase, the glue dispenser was placed in crushed ice when not in use to avoid the glue drying and blocking the tip. The labelling was performed without using anaesthetic on the bees.

The RFID tags were registered and allocated per treatment beforehand (cf. 4.3 RFID device). After labelling, the foragers were transferred in groups of 10 to 15 bees into cages (minimum of 3 cages of 10 bees per treatment). The cages with the RFID labelled bees were kept in the dark until the exposure phase.

Exposure phase: The test was conducted with 3 test item doses and one untreated control treatment **(geometric progression with a ratio of 3)**. The highest dose corresponded to a NOEL ('No Observed Effect Level') on mortality, 48-h after exposure.

Treatment	Test item doses
1	Untreated control (acetone 0.1 % (v/v))
2	0.11 ng per bee
3	0.33 ng per bee
4	1 ng per bee

Exposure procedure: The honeybees were exposed by feeding them with 20 µl per honeybee (200 µl per group of 10 bees) of the 30% (w/v) sucrose solution containing the test item 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 (e.g. the tip of a micropipette bevelled). The bees in each cage shared the feeding solution by trophallaxis. The exposure phase was completed once the honeybees had consumed all of the offered sucrose solution.

Exposure conditions: Maximum exposure duration was 1.5 hours. The start and end time of exposure were recorded for each cage. Sucrose solution consumption was regularly monitored. If at the end of the exposure period there was remaining sucrose solution in any of the test cage feeders, the feeders were weighed in order to further calculate the actual dose received per bee based on consumption. Feeders were weighed empty and full beforehand.

Post-exposure: The cages of honeybees which ingested all the administered feeding solution underwent an additional **one hour starvation period** in the dark.

Feeding before release: To take into account the time lag of sucrose solution consumption between the first and the last cages of bees during the exposure phase, each cage of bees **were fed *ad libitum*** with a 30 % (w/v) sucrose solution or with candy (e.g. Apifonda®) in 2016 and only with candy in 2017 after the **one hour post-exposure starvation** (**Figure 1**)**.** Time at which food was provided was recorded.

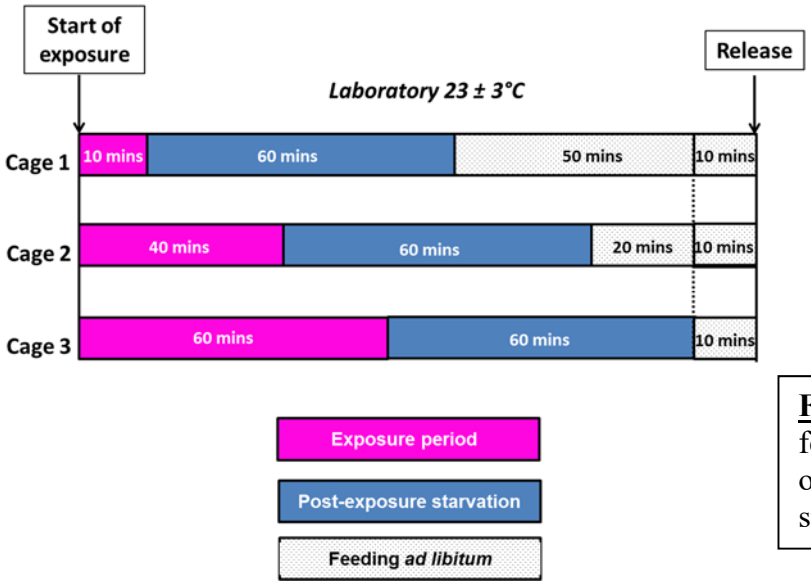


Figure 2: Illustration of the feeding procedure after the one hour post-exposure starvation period

Mortality and tags lost: At the end of the exposure phase, any dead honeybees and those that may have lost their tags, were recorded. They were collected during release phase to be identified thanks to the UID of the tag and thus could be excluded from any calculations.

From the end of the labelling phase to the release phase in the field, the number of dead bees was used to calculate **the mortality rate per treatment for each run**.

Honeybees release
Transport: The honeybees were transported **to the same site as they were released at the first time after powdering at 1 km (+/- 100 m) from the colony**.
Temperature and humidity levels during transport were maintained to ensure their safe keeping, particularly if the release place is far away from the laboratory (transport of the bee cages in cool boxes containing a damp cloth, in a box incubator…)

Before release: Release phase took place after the one hour post-exposure starvation period of the last cages that consumed all the sucrose solution. To homogenize exposure conditions of all the bees before release, the last cages of bees that finished the starvation period were supplied with food ad libitum for 10 minutes before release, whereas the food supply of the other bees continued (**Figure 2**).

Release: After this 10 minutes period, all cages of the test run were put in the same place, on a flat surface a few cms off the ground, and then opened simultaneously.

A period of at least two hours was ensured between the release time and sunset to allow foragers flying back to the hive.

Release start and end time (hour and minutes) were recorded. Local weather conditions (temperature and hygrometry (%)) were recorded during the release phase. Cloud cover and wind strength were also qualitatively estimated (**Appendix 8)**.

Climatic conditions: Weather conditions should be favourable for foraging activity (wind below 5 of Beaufort scale, temperatures of at least 15°C and no rain) when the honeybees were released.

4.8 Test Schedule

Bee powdering, capture, labelling, exposure and release phases for the test took place over one day.

The homing flight recording of the labelled foragers started immediately after the release and continued for 24 hours. This 24h-recording period was shown to be appropriate for assessing the homing success of released bees (Henry et al. 2012, homing flight ring test results 2015 to 2017).

4.9 Assessment

The data recorded with RFID readers for the bees returning to the hive: Following raw data were recorded in electronic form (MAJA Host storage system equipped with the appropriate software via PC connected to the host): the UID of the tag, the reader number and the reading time (date, hour, minute and second). Data were collected 24 hours after the release.

The weather conditions: temperature (T°C) and hygrometry (%) per hour were recorded using a data logger placed under the tested hive with RFID system. Rainfall (mm) per day was also recorded at the same place using a rain gauge.

4.10 Summary of the protocol changes from 2016 to 2017

	2016	**2017**
Quantity of colored powder (mg per bee)	0.5	0.3
Duration of the feeding phase *ad libitum* with candy before pre-exposure starvation	0.5 h	1 h
Type of feeding *ad libitum* before release	Candy or sucrose solution 30 % (w/v)	Candy

4.11 Results presentation and statistical analysis

- **Homing success and homing duration**

After labelling and before release in the field, the number of dead bees was used to calculate a mortality rate per treatment for each run.

Homing performance was characterised by two variables to explain:

- The homing flight success (**main variable**), which is a binomial variable with a value of 1 if the honeybee returns to the hive and is recorded over the 24-hours period, or 0 if it does not return.
- The homing time 24 hours after release (**secondary variable**), which is a quantitative variable. For each honeybee, it is defined as the time between the release and the first recording when entering the hive.

Homing success and its duration was determined from three files: first from the honeybees released (with the UID of the tags), second from the information at the release station (date, hour and minutes of release) and third from the RFID recording at the hive entrance. The three data sets were used to provide with R statistical software one raw data file per identified honeybee and treatment where homing time was expressed in minutes. During the 24 hours of RFID recording, a honeybee can be recorded several times when it passes the RFID reader (in or out the hive) for foraging activities. Therefore, several data points were recorded and can be calculated for a same bee. We only used the shortest homing time per bee which corresponds to the first recording at the hive after release. A bee which didn`t return to the hive after release was missing in the raw data, therefore was identified due to the missing tag code in the raw data when compared to the registered tags code before release.
The statistical analysis was performed using the software R version 3.3.1 (R Development Core Team, 2016). One raw data file was created per run and for the 3 runs pooled together for data analysis. Data from the 3 test runs were pooled to maximize the total number of bees per treatment (total of ≥ 90 bees labelled with a RFID tag) for the homing test analysis including data structuration and statistical treatments (Henry et al. 2012). The results are presented as cumulative homing probability to the hive over the 24-hours period per test item treatment and control group. Homing duration was illustrated as boxplots (medians, quartiles). Results of each test run are also presented.

From the results of the three test runs (pooled data), the homing rates to the hive obtained over the 24-hour period for each treatment were compared using a Chi^2 test ($P < 0.05$). An adjusted significance threshold was applied for paired comparisons with Bonferroni method. Concerning homing duration, data normality and homogeneity of variance were first tested with a Shapiro-Wilk test and a Bartlett test respectively ($P > 0.05$). As data didn't show normal distribution and/or variance homogeneity, homing durations obtained were compared between treatments using a non-parametric Kruskal-Wallis test ($P < 0.05$) followed by a Mann-Whitney test for paired comparisons..

From the test data analysis, we determined a 'No Observed Effect Dose' (NOED) on the homing flight. The NOED was expressed as **ng of the test item per honeybee**.

- **Analysis of homing results variability**

In order to assess the effects of different factors on the homing performance ($P< 0.05$), as temperatures recorded during the test and parasitic infestation (varroa), we used generalized linear mixed-effects models (GLMMs) with a logit link function using the R package lme4 (Bates et al., 2018). We considered data of all the labs together. The identity of experimental colonies of the ring test runs and of the release sites were included as random variables. The real exposure dose after analyses was introduced as a fixed, quantitative, explanatory variable. The additional explanatory variables were in one hand the temperature (ring tests 2016 and 2017) and in the other hand the varroa mite infestation (ring test 2017).
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 of size of effect and were comparable among each other. Data for varroa variable were log10-transformed.

4.12 Validity criterion of the study

Validity criterion was considered as the minimum and acceptable homing success rate of control bees for each test run. Based on the ring test results 2015, the minimum homing success rate was considered to be at least of 60% over the 24 hours period.

5-RESULTS AND DISCUSSION

Ten laboratories out of 11 in 2016 and 8 laboratories out of 11 in 2017 were able to perform the test. For some laboratories, it was not possible to perform the test or the 3 test runs because of lack of time, unfavourable climate. This result points out the interest of the powder method tested from 2016 to collect foragers familiar of the environment.

In 2016, Lab 1 performed two tests on two different sites, **one in peri-urban landscape (A) and one in rural landscape (B).**

In 2017, Lab 1 performed the test with respectively **10 bees exposed per cage (A) and 2 bees exposed per cage (B). Lab 4** didn't feed the bees *ad libitum* with candy before release **but with 200 µl for 10 bees of 30 % (w/v) sucrose solution**. This change followed the homing results of pre-tests 2017 performed by Lab 4 when feeding the bees *ad libitum* before release with candy or sucrose solution *ad libitum* (see part 5.7.3 Effect of a feeding period before release).

5.1 Mortality before release

The bee mortality was considered from the end of the exposure phase to the release phase in the field.

Tables 1 to 6 present numbers of foragers labelled and released in 2016 and 2017. Except for the run 1 of Lab 10 in 2016, the volumes of treated sucrose solution administered to groups of bees during exposure phase were totally consumed each time, for each treatment and run. As a whole, lower mortalities before release were experienced for a majority of laboratories in 2017 (Figures 6 to 8).

In 2016, mortality levels ranged from 0 to 60 % for the run 1 (Figure 3). Two laboratories (Lab 7 and 10) experienced high mortalities (Figure 3 and Table 1). Especially, Lab 10 experienced some problems to conduct the test and high number of bees was dying. For runs 2 and 3, mortality levels decreased and ranged from 0 to 28.3 % (Figures 4 and 5). Homing success of released bees was not impacted when mortality rates was higher than 20 % except for Lab 10 (Table 7).

In 2017, mortality levels were low for the runs 1 and 3 and ranged from 0 to 7.6 % (run 1, Figure 6) and from 0 to 13.3 % (run 3, Figure 8).
For the test run 2, higher mortalities were recorded after the exposure to the 1-ng dose for Lab 6 (65 %) and 7 (22.5 %) (Figure 7).

Table 1: Number of labelled (LB) and released bees (RB) for the test run 1 in 2016

Lab	Bee race	Nb of bees	Control	0.11 ng/bee	0.33 ng/bee	1 ng/bee
1A*	Carnica	LB	30	30	30	30
		RB	28	28	29	28
1B*	Carnica	LB	30	30	30	30
		RB	30	30	30	29
2	Ligustica	LB	35	35	35	35
		RB	34	34	34	35
3	Carnica	LB	40	40	40	40
		RB	37	39	40	39
4	Buckfast	LB	30	30	30	30
		RB	29	28	27	25
5	Carnica	LB	42	42	42	42
		RB	41	41	41	40
6	Buckfast	LB	30	30	30	30
		RB	30	30	30	30
7	Carnica	LB	30	30	30	30
		RB	23	27	22	20
8	Ligustica	LB	30	30	30	30
		RB	29	28	28	28
9	Black x Buckfast	LB	40	39	40	40
		RB	35	33	36	36
10	Carnica x Buckfast	LB	40	40	40	40
		RB	15	21	31	27

*Lab 1 performed two tests on two different sites, one in peri-urban landscape (**A**) and one in rural landscape (**B**).

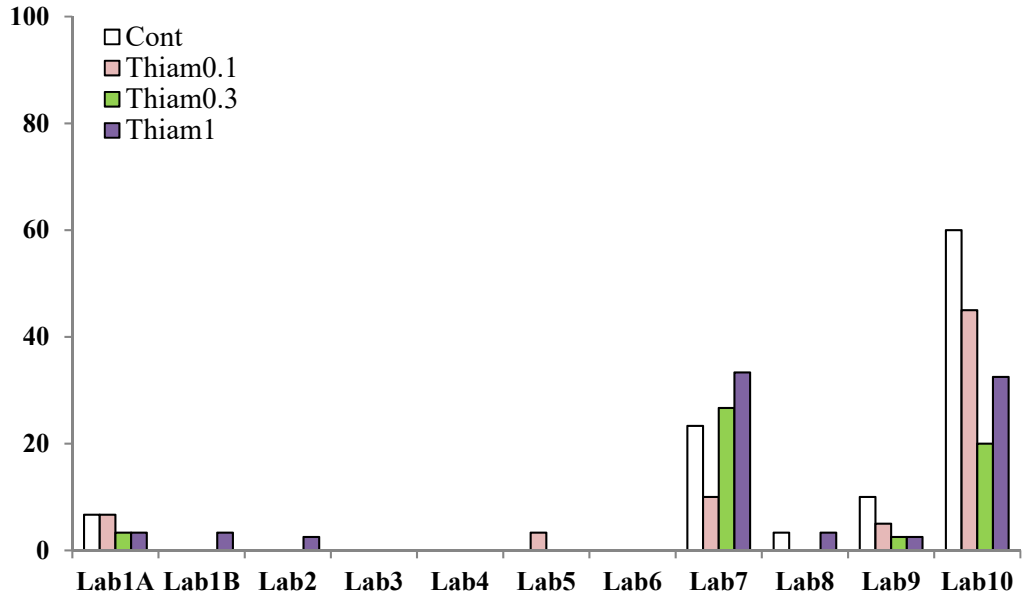


Figure 3: Mortality rate (%) before release for the test run 1 in 2016

Table 2: Number of labelled (LB) and released bees (RB) for the test run 2 in 2016

Lab	Bee race	Nb of bees	Control	0.11 ng/bee	0.33 ng/bee	1 ng/bee
1A*	Carnica	LB	40	40	40	40
		RB	39	39	37	33
1B*	Carnica	LB	30	30	30	30
		RB	28	29	29	29
2	Ligustica	LB	39	40	40	40
		RB	38	32	33	29
3	Carnica	LB	30	30	30	30
		RB	30	29	28	28
4	Buckfast	LB	30	30	30	30
		RB	29	30	29	30
5	Carnica	LB	42	42	42	42
		RB	42	40	41	41
6	Buckfast	LB	30	30	30	30
		RB	30	30	30	30
7	Carnica	LB	30	30	30	30
		RB	28	26	28	27
8	Ligustica	LB	30	30	30	30
		RB	27	22	26	21
9	Black x Buckfast	LB	23	23	23	23
		RB	22	20	21	21
10	Carnica x Buckfast	LB	40	40	40	40
		RB	37	34	35	32

*Lab 1 performed two tests on two different sites, one in peri-urban landscape (**A**) and one in rural landscape (**B**).

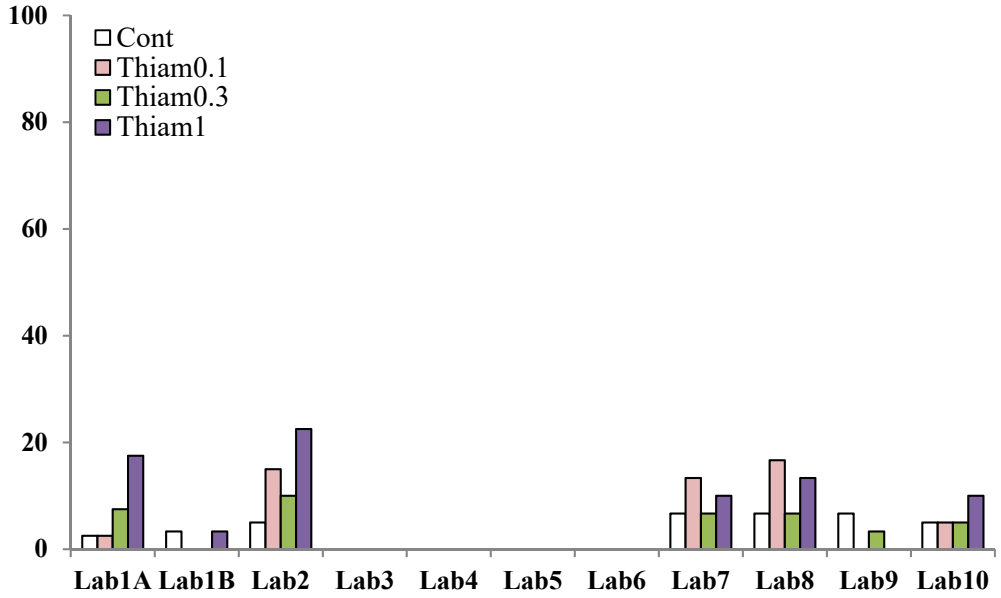


Figure 4: Mortality rate (%) before release for the test run 2 in 2016

Table 3: Number of labelled (LB) and released bees (RB) for the test run 3 in 2016

Lab	Bee race	Nb of bees	Control	0.11 ng/bee	0.33 ng/bee	1 ng/bee
1A*	Carnica	LB	30	30	30	30
		RB	30	29	30	29
1B*	Carnica	LB	30	30	30	30
		RB	30	29	30	29
2	Ligustica	LB	40	40	40	39
		RB	36	39	35	25
3	Carnica	LB	40	40	40	40
		RB	40	40	40	40
4	Buckfast	LB	30	30	30	30
		RB	30	28	29	30
5	Carnica	LB	42	42	42	42
		RB	38	42	41	38
6	Buckfast	LB	30	30	30	30
		RB	30	30	30	30
7	Carnica	LB	30	30	30	30
		RB	29	30	30	28
9	Black x Buckfast	LB	40	39	40	40
		RB	38	38	39	34

*Lab 1 performed two tests on two different sites, one in peri-urban landscape (**A**) and one in rural landscape (**B**).

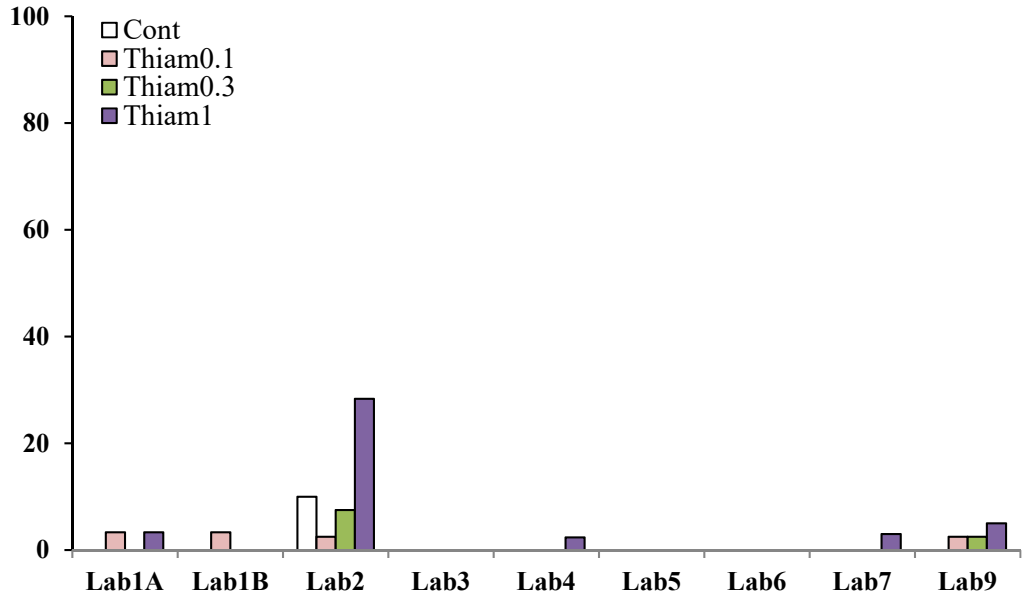


Figure 5: Mortality rate (%) before release for the test run 3 in 2016

Table 4: Number of labelled (LB) and released bees (RB) for the test run 1 in 2017

Lab	Bee race	Nb of bees	Control	0.11 ng/bee	0.33 ng/bee	1 ng/bee
1A*	Carnica	LB	30	30	30	30
		RB	30	30	30	30
1B*	Carnica	LB	30	30	30	30
		RB	30	30	30	30
2	Ligustica	LB	40	40	40	40
		RB	33	37	35	36
3	Carnica	LB	40	40	40	40
		RB	40	37	37	39
4**	Buckfast	LB	30	30	30	30
		RB	30	28	30	30
5	Ligustica	LB	30	30	30	30
		RB	22	27	20	24
6	Black x Buckfast	LB	40	40	40	40
		RB	38	37	38	36
7	Carnica x Buckfast	LB	40	40	40	40
		RB	39	38	39	40
8	Buckfast	LB	30	30	30	30
		RB	30	30	30	30

*Lab 1 performed 2 tests with (**A**) a group of 10 bees exposed per cage and (**B**) with 2 bees exposed per cage

**Lab 4 did not feed the bees *ad libitum* with candy before release but with 200 µl for 10 bees of 30 % (w/v) sucrose solution. This change followed the homing results of pre-tests 2017 performed by Lab 4 when feeding the bees *ad libitum* before release with candy or sucrose solution (see part 5.7.3 Effect of a feeding period before release).

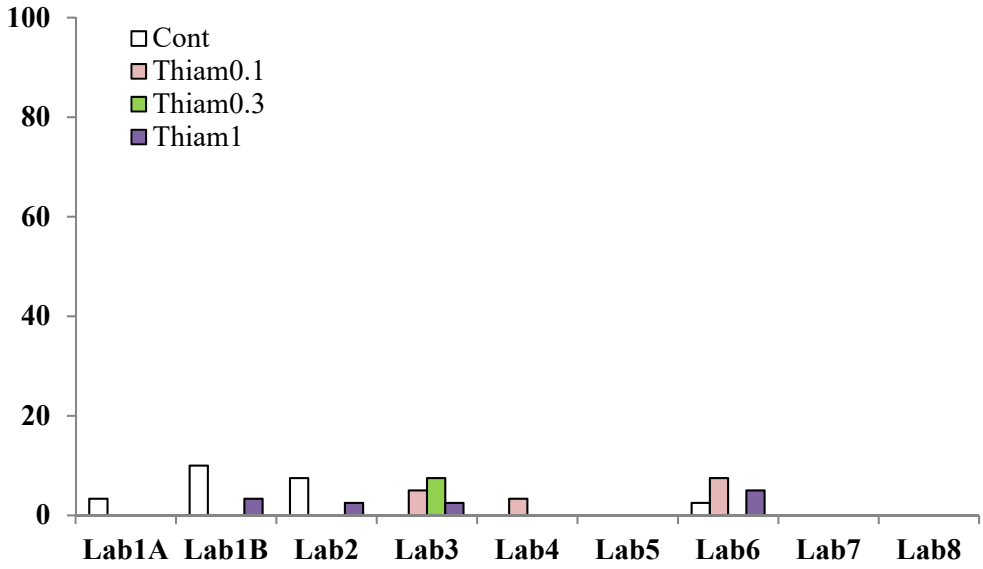


Figure 6: Mortality rate (%) before release for the test run 1 in 2017

Table 5: Number of labelled (LB) and released bees (RB) for the test run 2 in 2017

Lab	Bee race	Nb of bees	Control	0.11 ng/bee	0.33 ng/bee	1 ng/bee
1A*	Carnica	LB	30	30	30	30
		RB	30	30	30	30
1B*	Carnica	LB	30	30	30	30
		RB	30	30	30	30
2	Ligustica	LB	40	40	40	40
		RB	38	40	38	34
3	Carnica	LB	40	40	40	40
		RB	38	39	40	40
4	Buckfast	LB	30	30	30	30
		RB	28	29	26	28
5	Ligustica	LB	30	30	30	30
		RB	26	30	27	26
6	Black x Buckfast	LB	40	40	40	40
		RB	29	38	31	10
7	Carnica x Buckfast	LB	40	40	40	40
		RB	38	35	38	31

*Lab 1 performed 2 tests with (**A**) a group of 10 bees exposed per cage and (**B**) with 2 bees exposed per cage

**Lab 4 did not feed the bees ad libitum with candy before release but with 200 µl for 10 bees of 30 % (w/v) sucrose solution. This change followed the homing results of pre-tests 2017 performed by Lab 4 when feeding the bees *ad libitum* before release with candy or sucrose solution (see part 5.7.3 Effect of a feeding period before release).

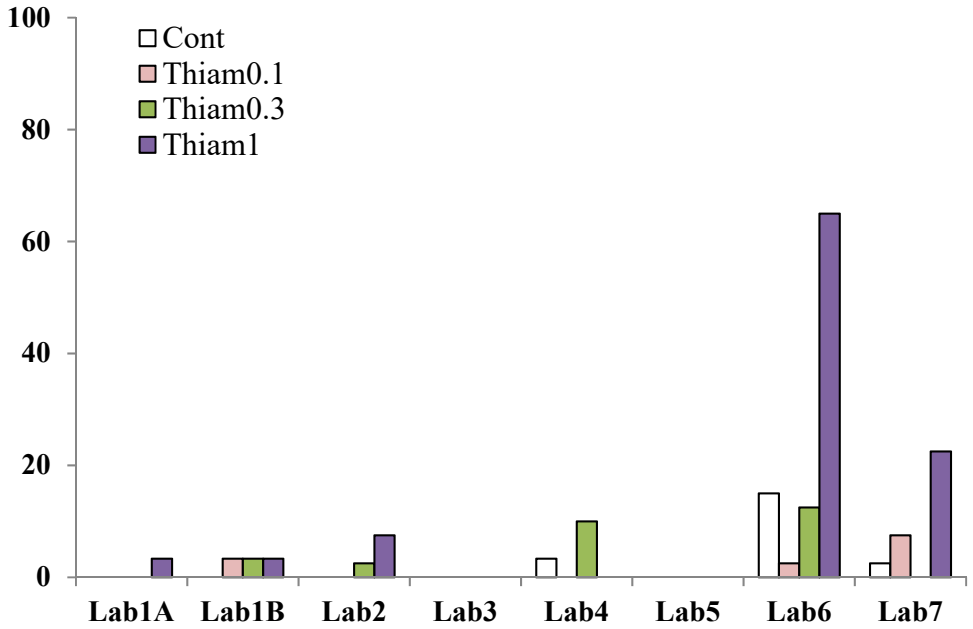


Figure 7: Mortality rate (%) before release for the test run 2 in 2017

Table 6: Number of labelled (LB) and released bees (RB) for the test run 3 in 2017

Lab	Bee race	Nb of bees	Control	0.11 ng/bee	0.33 ng/bee	1 ng/bee
1A*	Carnica	LB	30	30	30	30
		RB	30	30	30	30
1B*	Carnica	LB	30	30	30	30
		RB	30	30	30	30
2	Ligustica	LB	40	40	40	40
		RB	37	40	38	38
3	Carnica	LB	30	30	30	30
		RB	30	30	30	30
4	Buckfast	LB	30	30	30	30
		RB	28	30	29	30
5	Ligustica	LB	30	30	30	30
		RB	28	30	27	25
6	Black x Buckfast	LB	30	30	30	30
		RB	25	27	29	25
7	Carnica x Buckfast	LB	40	40	40	40
		RB	40	38	40	38

*Lab 1 performed 2 tests with (**A**) a group of 10 bees exposed per cage and (**B**) with 2 bees exposed per cage

**Lab 4 did not feed the bees ad libitum with candy before release but with 200 µl for 10 bees of 30 % (w/v) sucrose solution. This change followed the homing results of pre-tests 2017 performed by Lab 4 when feeding the bees *ad libitum* before release with candy or sucrose solution (see part 5.7.3 Effect of a feeding period before release).

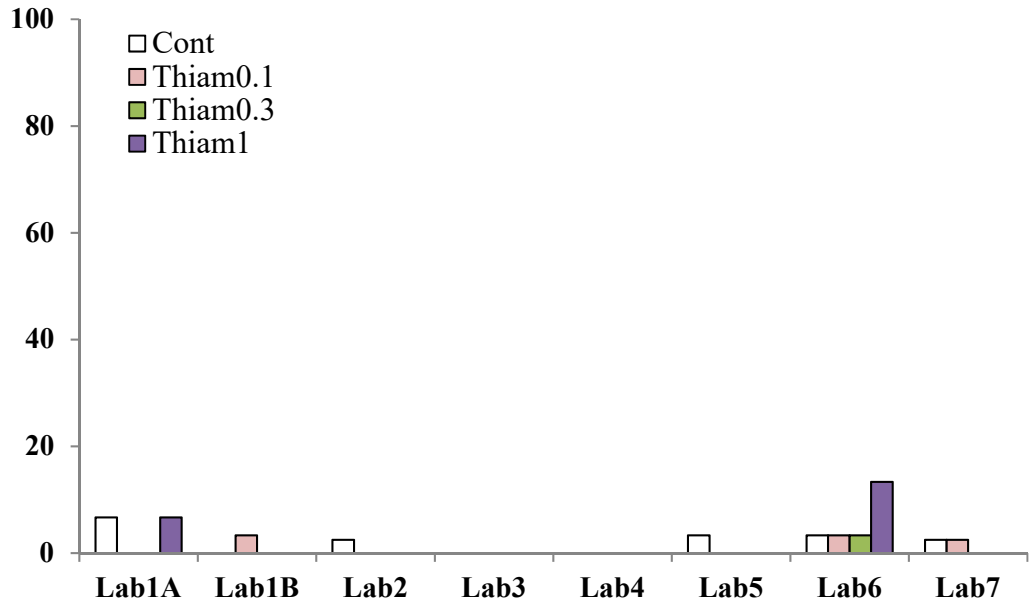


Figure 8: Mortality rate (%) before release for the test run 3 in 2017

5.2 Homing success per treatment and run

A total of 31 and 25 test runs were conducted as a whole by all participating laboratories in 2016 and 2017 respectively. For each treatment, results concerning homing success 24 h-after release showed variability from one test run to another as well as within each lab (Tables 7 and 8). For a majority of labs, homing performances of control bees was more important than the minimum and acceptable rate of 60% for both years. Some runs had lower homing performances for control bees. This represents 6 runs on 31 (19%) in 2016 but only 3 runs on 25 (12 %) in 2017.

For a majority of runs, results show lower homing performances for the bees exposed to the highest dose compared to control bees or bees exposed to lowest doses for both years (Tables 7 and 8; Figure 9). However, for 11 runs on 31 (35.5 %), only 10 % or less homing differences were obtained between control bees and bees exposed to the highest dose in 2016 (Table 7). In 2017, 7 runs on 25 (28 %) showed 10 % or less homing differences between control bees and bees exposed to the highest dose (Table 8). Without the results of Lab B with another mode of exposure tested, it decreased to 5 runs on 22 (22.7 %).

Considering only valid tests (homing performances of control bees ≥ 60%), 9 runs on 29 (31 %) showed only 10 % or less homing differences between control bees and bees exposed to the highest dose in 2016 (Table 4). In 2017, it decreased to 5 runs on 23 (21.7 %). Without the results of Lab B with another mode of exposure tested, only 3 resting runs on 20 (15 %) were concerned.

We note that some real doses analysed in 2017 were extremely high, especially for the 1-ng expected tested dose compared to 2016 (Figure 9, see also 5.4 Analyses of the test item solutions).

Table 7: Results of homing probabilities per test run and laboratory in 2016

Lab	Run	Control	Thiam. 0.1ng/bee	Thiam. 0.3 ng/bee	Thiam. 1 ng/bee
1A[a]	**1**	**0.964**	**0.929**	**1.000**	**1.000**
	2	**0.923**	**0.949**	**0.973**	**0.818**
	3	0.933	0.931	0.967	0.793
1B[a]	**1**	**0.967**	**1.000**	**0.967**	**0.931**
	2	1.000	0.931	1.000	0.793
	3	**1.000**	**1.000**	**0.967**	**0.897**
2	**1**	0.706	0.676	0.676	0.343
	2	0.711	0.688	0.697	0.517
	3	0.611	0.615	0.543	0.240
3	**1**	0.811	0.692	0.750	0.538
	2	1.000	0.966	0.571	0.571
	3	**0.975**	**0.950**	**1.000**	**0.950**
4	**1**	0.621	0.679	0.481	0.360
	2	**0.690**	**0.767**	**0.793**	**0.867**
	3	**0.667**	**0.750**	**0.759**	**0.833**
5	**1**	**0.927**	**0.878**	**0.927**	**0.850**
	2	**0.833**	**0.850**	**0.780**	**0.780**
	3	0.868	0.976	0.878	0.711
6	***1**	*0.567*	*0.567*	*0.600*	*0.167*
	***2**	*0.233*	*0.500*	*0.400*	*0.133*
	3	0.800	0.767	0.967	0.667
7	**1**	1.000	0.889	0.863	0.450
	2	0.714	0.808	0.857	0.407
	3	0.862	0.767	0.800	0.179
8	***1**	***0.517***	***0.643***	***0.500***	***0.679***
	2	0.667	0.591	0.500	0.286
9	**1**	0.771	0.636	0.694	0.472
	***2**	*0.591*	*0.750*	*0.524*	*0.381*
	3	0.789	0.816	0.872	0.471
10	***1**	*0.533*	*0.286*	*0.323*	*0*
	***2**	*0.135*	*0.206*	*0.200*	*0.031*

***** Tests not valid because of low homing success of control bees (< 60%)

Runs with no or low differences in homing success between 1ng-exposed bees and control bees (≤ 10 % of homing success between control and 1ng-bees) or homing success of 1ng-bees more important than control bees.

[a] Lab 1 performed two tests on two different sites, one in peri-urban landscape (**A**) and one in rural landscape (**B**).

Table 8: Results of homing probabilities per test run and laboratory in 2017

Lab	Run	Control	Thiam. 0.1ng/bee	Thiam. 0.3 ng/bee	Thiam. 1 ng/bee
1A[a]	**1**	0.933	0.933	0.867	0.767
	2	0.867	0.867	0.833	0.333
	3	0.767	0.767	0.633	0.567
1B[a]	**1**	**0.833**	**0.933**	**0.967**	**0.800**
	2	**0.833**	**0.967**	**0.833**	**0.800**
	3	0.933	0.833	0.867	0.767
2	**1**	0.636	0.676	0.514	0.167
	2	0.632	0.575	0.789	0.294
	3	0.649	0.650	0.711	0.421
3	**1**	0.850	0.892	0.757	0.718
	2	0.868	0.974	0.875	0.750
	3	0.967	0.733	0.933	0.700
4[b]	**1**	**0.867**	**0.786**	**0.767**	**0.933**
	2	0.893	0.690	0.962	0.679
	3	0.857	0.967	0.862	0.733
5	***1**	**0.545**	**0.481**	**0.555**	**0.625**
	***2**	**0.577**	**0.667**	**0.815**	**0.538**
	3	0.750	0.833	0.852	0.280
6	**1**	**0.763**	**0.730**	**0.868**	**0.667**
	2	0.828	0.816	0.839	0.400
	3	**0.760**	**0.926**	**0.862**	**0.760**
7	**1**	0.923	0.921	0.897	0.750
	2	0.895	0.829	0.947	0.129
	3	0.875	0.658	0.825	0.211
8	***1**	0.567	0.667	0.200	0.333

***** Tests not valid because of low homing success of control bees (< 60%)

▢ Runs with no or low differences in homing success between 1ng-exposed bees and control bees (≤ 10 % of homing success between control and 1ng-bees) or homing success of 1ng-bees more important than control bees.

[a] Lab 1 performed 2 tests with (**A**) a group of 10 bees exposed per cage and (**B**) with 2 bees exposed per cage

[b] Lab 4 did not feed the bees ad libitum with candy before release but with 200 µl per 10 bees of 30 % (w/v) sucrose solution. This change followed the homing results of pre-tests 2017 performed by Lab 4 when feeding the bees *ad libitum* before release with candy or sucrose solution (see part 5.7.3 Effect of a feeding period before release).

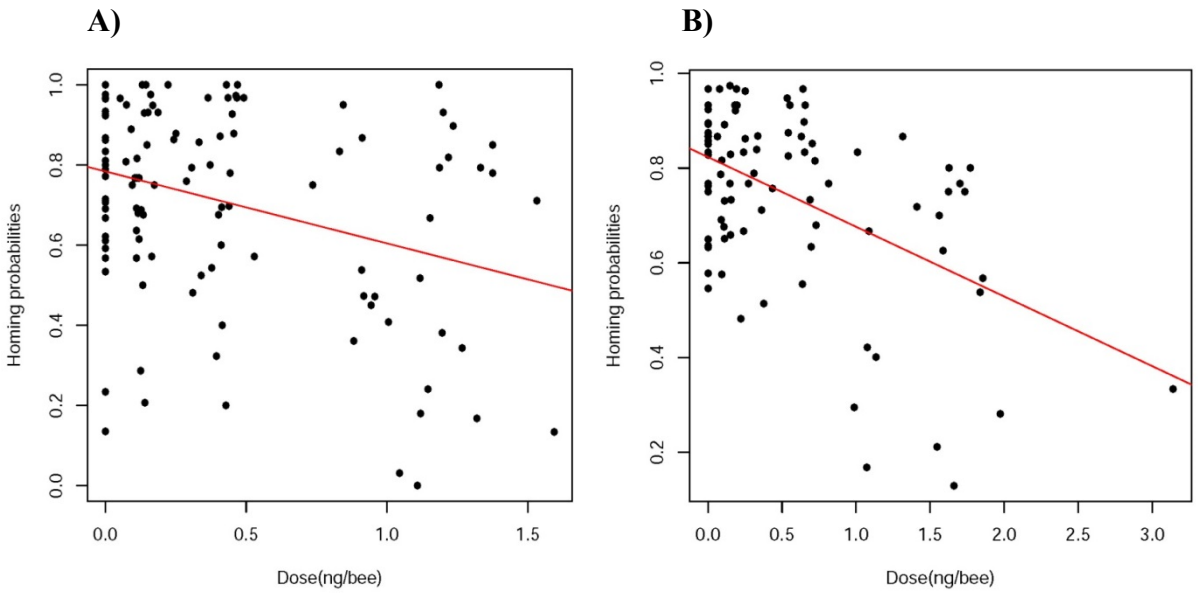


Figure 9: Relationships between homing probabilities of the foragers 24 hours after release and the real doses of exposure (ng per bee) after analyses for all the laboratories and test runs. For 2017, results of Lab 8 (run 1) and Lab 6 (run 3) are not considered as the analyses of the real doses were not performed. A red regression line was added. **A) results obtained in 2016**, **B) results obtained in 2017**.

5.3 Climatic conditions during the tests

Climatic conditions recorded during the homing flight tests 24h-after bees' release are presented in Tables 9 and 10 for 2016 and 2017 respectively.

Table 9: Mean climatic conditions recorded during 24 hours after bees' release for each laboratory and run in 2016

Lab	Run	Mean temperature (°C)	Mean hygrometry (%)	Mean rainfall (mm)
1A	**1**	18.15	55.66	0
	2	18.68	53.30	15[a]
	3	18.81	60.66	0
1B	**1**	24.33	60.44	0
	2	20.61	59.75	0
	3	18.32	76.08	19.9[a]
2	**1**	18.62	82.80	0.1
	2	21.77	71.52	0.1
	3	20.92	79.50	8[b]
3	**1**	19.71	60.02	0
	2	20.76	70.47	0
	3	24.97	53.26	0
4	**1**	27.96	49.94	0
	2	28.82	56.08	0
	3	29.21	47.65	0
5	**1**	23.73	68.35	0.25
	2	25.62	70.96	0.75
	3	18.13	68.40	1.25
6	***1**	*17.18*	*86.76*	*0*
	***2**	*18.28*	*83.86*	*0*
	3	20.06	77.81	0
7	**1**	15.18	83.84	0
	2	18.26	69.88	0
	3	15.62	75.24	0
8	***1**	*22.76*	*73.35*	*1.2*
	2	22.34	74.31	9.8
9	**1**	19.35	61.79	0
	***2**	*20.10*	*75.20*	*0*
	3	15.58	70.55	0
10	***1**	*19.85*	*64.86*	*0*
	***2**	*21.67*	*62.89*	*0*

* Tests not valid because of low homing success of control bees (< 60%)

[a] Heavy rain during the night

[b] Rain the second day after release

Table 10: Mean climatic conditions recorded during 24 hours after bees' release for each laboratory and run in 2017

Lab	Run	Mean temperature (°C)	Mean hygrometry (%)	Mean rainfall (mm)
1A[a]	**1**	21.11	60.34	0
	2	27.18	56.03	0
	3	29.23	52.66	0
1B[a]	**1**	26.02	53.09	0
	2	-	-	0
	3	-	-	0
2	**1**	21.12	63.70	0
	2	27.16	55.82	0
	3	20.08	78.04	0.5
3	**1**	20.92	80.66	0
	2	18.10	70.16	0.1
	3	19.70	69.73	0
4[b]	**1**	29.04	46.46	0
	2	30.12	53.06	0
	3	31.00	57.44	0
5	***1**	26.43	58.04	9.8[c]
	***2**	27.49	68.52	1
	3	27.57	49.08	6.6[c]
6	**1**	24.20	66.41	1.5
	2	20.79	57.13	0
	3	21.41	74.94	0
7	**1**	26.33	70.59	0
	2	21.99	73.63	0
	3	23.40	67.98	0
8	***1**	22.35	73.21	0

***** Tests not valid because of low homing success of control bees (< 60%)

[a] Lab 1 performed 2 tests with (**A**) a group of 10 bees exposed per cage and (**B**) with 2 bees exposed per cage

[b] Lab 4 did not feed the bees ad libitum with candy before release but with 200 µl per 10 bees of 30 % (w/v) sucrose solution. This change followed the homing results of pre-tests 2017 performed by Lab 4 when feeding the bees *ad libitum* before release with candy or sucrose solution (see part 5.7.3 Effect of a feeding period before release).

[c] rain during night

In 2016, fresh mean temperatures on the 24-h period were recorded for lab 7 (runs 1 and 3) and lab 9 (run 3) but they didn't impact the homing performances of the bees. High homing success rate of control bees was recorded with about 80 to 100 %. In the same manner, significant rainfall was recorded especially for lab 1 (A and B). However, rain occurred during night and didn't impact the homing success of the bees.

Climatic conditions cannot explain low homing performances for some test runs, where homing rate of control bees < 60 %, because no fresh or abnormal temperatures were recorded (Table 9).

In 2017, climatic conditions were favorable for all the labs as a whole. Important rainfalls were recorded especially for Lab 5 but they occurred during night and didn't impact the homing success of the bees (Table 10).

5.4 Analyses of the test item solutions

The sucrose solutions were analysed by ANSES laboratory (Sophia Antipolis, France) using the liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) technique to detect real doses of thiamethoxam item (limit of thiamethoxam quantification = 0.3 ng/mL).

Results obtained in 2016 are presented in Table 11. Analyses were not performed for Lab 8.

Table 11: Results of the analytical analyses of the contaminated test sucrose solution for each test run and laboratory in 2016

Lab	Run	Nominal dose : 1 ng/bee	Nominal dose : 0.33 ng/bee	Nominal dose : 0.11 ng/bee
1A	**1**	1.184	0.43	0.138
	2	1.218	0.464	0.168
	3	1.332	0.492	0.186
1B	**1**	1.198	0.436	0.144
	2	1.186	0.468	0.152
	3	1.234	0.466	0.130
2	**1**	1.266	0.402	0.134
	2	1.116	0.438	0.128
	3	1.144	0.378	0.120
3	**1**	0.910	**0.736**	0.110
	2	**0.528**	0.164	**0.052**
	3	0.844	0.222	0.074
4	**1**	0.882	0.310	0.116
	2	0.912	0.306	0.112
	3	0.830	0.288	0.096
5	**1**	1.374	0.450	0.250
	2	1.374	0.442	0.148
	3	**1.530**	0.456	0.160
6	***1**	1.318	0.410	0.110
	***2**	**1.592**	0.414	0.132
	3	1.152	0.364	0.104
7	**1**	0.942	0.242	0.092
	2	1.004	0.332	0.072
	3	1.118	0.372	0.120
9	**1**	0.916	0.412	0.110
	***2**	1.194	0.340	0.174
	3	0.956	0.408	0.112
10	***1**	1.108	0.394	0.126
	***2**	1.044	0.428	0.140

***** Tests not valid because of low homing success of control bees (< 60%)

In 2017, analysis performed showed some high and/or abnormal values for several labs. We especially noted 7 outliers highlighted in red (Table 12). Mean doses values stayed high compared to nominal doses expected. Something may have gone wrong as there is no correlation between analytical results and mortality or homing performances results.

Table 12: Results of the analytical analyses of the contaminated test sucrose solution for each test run and laboratory in 2017

Lab	Run	Nominal dose : 1 ng/bee	Nominal dose : 0.33 ng/bee	Nominal dose : 0.11 ng/bee
1A	1	1.700	0.628	0.182
	2	**3.138**	**1.008**	**1.314**
	3	1.854	0.696	**0.812**
1B	1	1.770	0.640	0.194
	2	1.626	0.652	0.192
	3	**0.146**	**0.064**	**0**
2	1	1.070	0.374	0.106
	2	0.988	0.308	0.092
	3	1.076	0.362	0.110
3	1	1.408	0.436	0.110
	2	1.622	0.542	0.146
	3	1.562	0.554	0.154
4	1	0.656	0.272	0.084
	2	0.728	0.252	0.088
	3	0.690	0.252	0.078
5	*1	1.588	0.638	0.220
	*2	1.836	0.720	0.238
	3	1.972	0.702	0.238
6	1	1.084	0.336	0.110
	2	1.134	0.328	0.092
7	1	1.732	0.648	0.184
	2	1.658	0.534	0.150
	3	1.546	0.542	0.150

* Tests not valid because of low homing success of control bees (< 60%)

5.5 Homing success per treatment

To assess homing success per treatment, data of the three test runs were pooled when homing performance of control bees were ≥ 60 % in individual test runs. Results of test 2 performed by Lab 9 were kept as homing result of control bees (59 %) was very close to the validity criterion.

In 2016, bees exposed to the highest dose of thiamethoxam at 1 ng per bee returned to the hive at a significantly lower rate compared to control bees or to bees exposed at least to 0.11 ng per bee for 5 labs out of 8 (Chi^2 or Fisher's Exact tests; $P < 0.05$; Table 13, and Figure 10). For 3 tests out of 8, no significant differences were found between control bees and bees exposed to thiamethoxam (Chi^2 or Fisher's Exact tests; $P > 0.05$; Table 13, and Figure 11).

Then, a nominal No Observed Effect Dose (NOED) on the homing success of 0.33 ng per bee for 5 labs out of 8 could be determined.

Table 13: Homing success results for the ring test 2016 (three valid test runs pooled)

		Control	Thiam. 0.11 ng/bee	Thiam. 0.33 ng/bee	Thiam. 1 ng/bee
Lab 1A	Number of foragers released	97	96	96	90
	Homing success probability (24 h after release) [1]	0.938 (ab)	0.938 (ab)	0.979 (a)	0.867(b)
	Fisher's Exact Test	P = 0.027			
Lab 1B	Number of foragers released	88	88	89	87
	Homing success probability (24 h after release) [1]	**0.989 (a)**	**0.977 (ab)**	**0.978 (ab)**	**0.874 (b)**
	Fisher's Exact Test	P = 0.0016			
Lab 2	Number of foragers released	108	105	102	89
	Homing success probability (24 h after release) [1]	**0.989 (a)**	**0.657(a)**	**0.637(a)**	**0.371(b)**
	Chi² Test	Chi² = 23.921, df = 3, P = 2.594e-05			
Lab 3	Number of foragers released	107	108	108	107
	Homing success probability (24 h after release) [1]	**0.925 (a)**	**0.861 (a)**	**0.796 (ab)**	**0.701 (b)**
	Chi² Test	Chi² = 20.031, df = 3, P = 0.0001672			
Lab 4	Number of foragers released	88	86	85	85
	Homing success probability (24 h after release) [1]	0.659 (a)	0.733 (a)	0.682 (a)	0.706 (a)
	Chi² Test	Chi² = 1.219, df = 3, P = 0.7485			
Lab 5	Number of foragers released	121	123	123	119
	Homing success probability (24 h after release) [1]	0.876 (a)	0.902 (a)	0.862 (a)	0.782 (a)
	Chi² Test	Chi²= 7.934, df = 3, P = 0.0474			
Lab 7	Number of foragers released	80	83	80	75
	Homing success probability (24 h after release) [1]	**0.850 (a)**	**0.819 (a)**	**0.838 (a)**	**0.333 (b)**
	Chi² Test	Chi²= 71.383, df = 3, P = 2.158e-15			
Lab 9	Number of foragers released	95	91	96	91
	Homing success probability (24 h after release) [1]	**0.737 (a)**	**0.736 (a)**	**0.729 (a)**	**0.451 (b)**
	Chi² Test	Chi² = 24.831, df = 3, P = 1.675e-05			

[1]Pairwise comparisons were performed with Fisher's exact test or Chi² test and used Bonferroni P value adjustment method. Same letters indicate no significant differences.

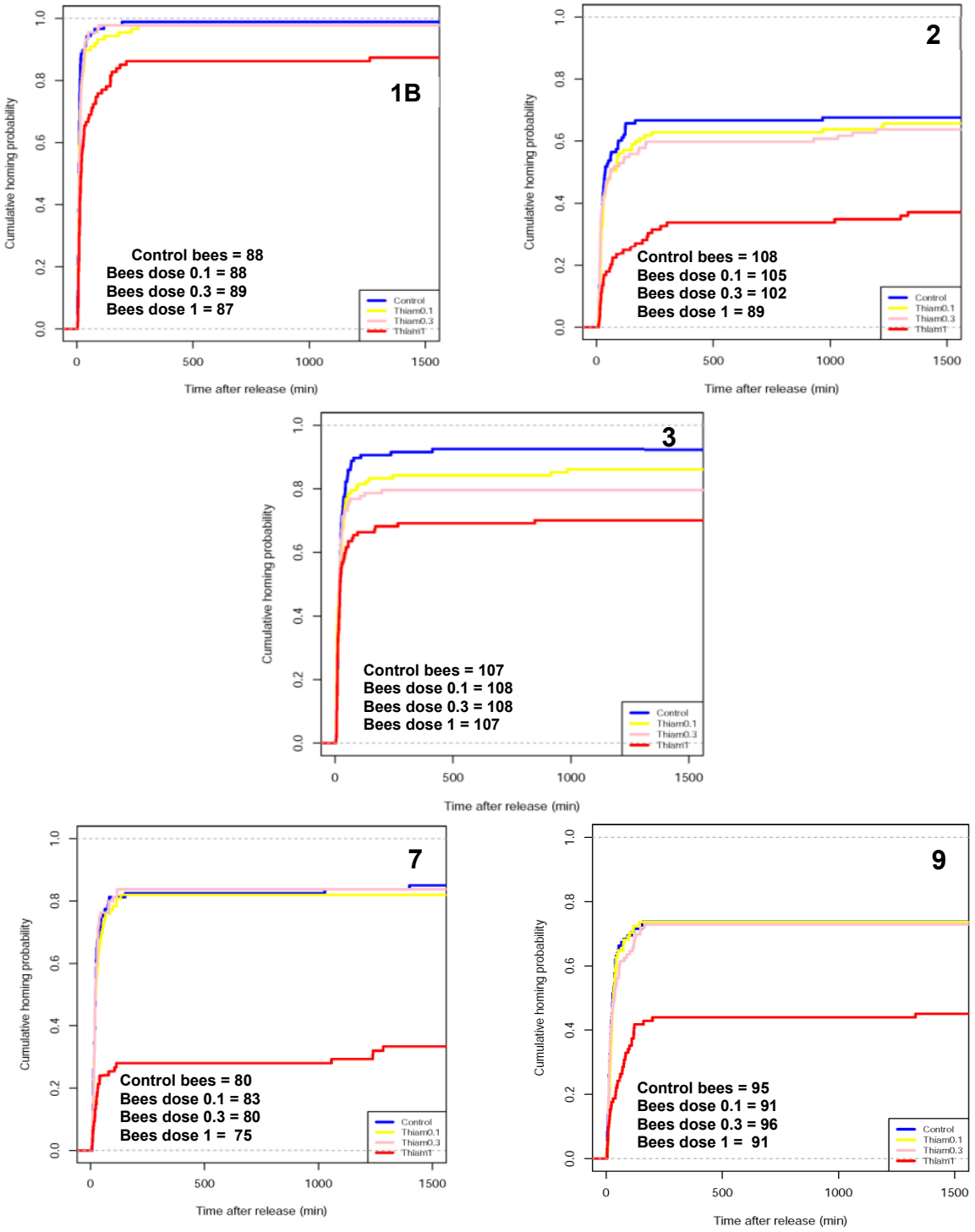


Figure 10: Cumulative homing probability of groups of foragers during 24 hours after release in 2016 for labs where a significant difference was found at least between control bees, bees exposed to 0.1 ng and 1-ng exposed bees (3 test runs pooled, Chi² tests, $P < 0.05$). The yellow curve represents homing performances for foragers exposed to 0.11 ng per bee of thiamethoxam, the pink curve for the 0.33 ng per bee treatment, the red curve for the 1 ng per bee treatment and the blue curve for control bees.

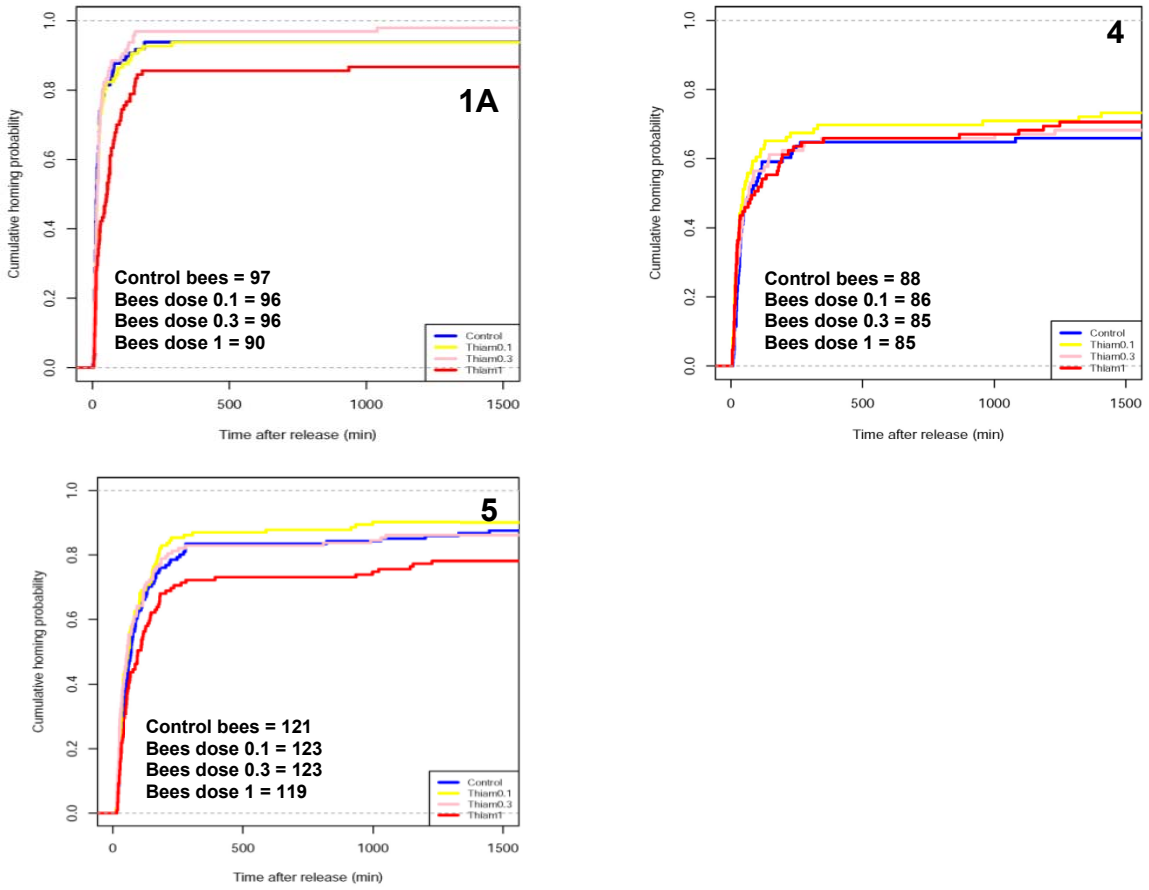


Figure 11: Cumulative homing probability of groups of foragers during 24 hours after release in 2017 for the labs where no significant differences were found between the different groups of bees (3 test runs pooled, Chi² tests, $P > 0.05$). The yellow curve represents homing performances for foragers exposed to 0.11 ng per bee of thiamethoxam, the pink curve for the 0.33 ng per bee treatment, the red curve for the 1 ng per bee treatment and the blue curve for control bees.

In 2017, bees exposed to the highest dose of thiamethoxam at 1 ng per bee returned to the hive at a significantly lower rate compared to control bees or to bees exposed at least to 0.11 ng per bee for 4 labs out of 7 (Chi² tests; $P < 0.05$; Table 14 and Figure 12). For 3 tests out of 7, no significant differences were found between exposed and control bees (Chi² tests; $P > 0.05$; Table 14, and Figure 13).

Considering only group of 10-exposed bees, a nominal No Observed Effect Dose (NOED) on the homing success of 0.33 ng per bee could be determined for 4 labs out of 6.

Table 14: Homing success results for the ring test 2017 (three valid test runs pooled)

		Control	Thiam. 0.11 ng/bee	Thiam. 0.33 ng/bee	Thiam. 1 ng/bee
Lab 1A	Number of foragers released	90	90	90	90
	Homing success probability (24 h after release) [1]	**0.856 (a)**	**0.856 (a)**	**0.778 (a)**	**0.556 (b)**
	Chi² Test	Chi²= 29.883, df = 3, P = 1.461e-06			
Lab 1B*	Number of foragers released	90	90	90	90
	Homing success probability (24 h after release) [1]	0.867 (a)	0.911 (a)	0.889 (a)	0.789 (a)
	Chi² Test	Chi²= 6.497, df = 3, P = 0.0898			
Lab 2	Number of foragers released	108	117	111	108
	Homing success probability (24 h after release) [1]	**0.639 (a)**	**0.632 (a)**	**0.676 (a)**	**0.296 (b)**
	Chi² Test	Chi² = 41.777, df = 3, P = 4.474e-09			
Lab 3	Number of foragers released	108	106	107	109
	Homing success probability (24 h after release) [1]	**0.889 (a)**	**0.877 (ab)**	**0.850 (ab)**	**0.725 (b)**
	Chi² Test	Chi² = 13.448, df = 3, P = 0.003762			
Lab 4	Number of foragers released	86	87	85	88
	Homing success probability (24 h after release) [1]	0.872 (a)	0.816 (a)	0.859 (a)	0.784 (a)
	Chi² Test	Chi² = 3.034, df = 3, P = 0.3864			
Lab 6	Number of foragers released	92	102	98	71
	Homing success probability (24 h after release) [1]	0.783 (ab)	0.814 (ab)	0.857 (a)	0.662 (b)
	Chi² Test	Chi² = 9.971, df = 3, P = 0.01882			
Lab 7	Number of foragers released	117	111	117	109
	Homing success probability (24 h after release) [1]	**0.897 (a)**	**0.802 (a)**	**0.889 (a)**	**0.385 (b)**
	Chi² Test	Chi² = 104.190, df = 3, P < 2.2e-16			

[1]Pairwise comparisons were performed with Chi² test and used Bonferroni P value adjustment method. Same letters indicate no significant differences.

*2 bees exposed per cage

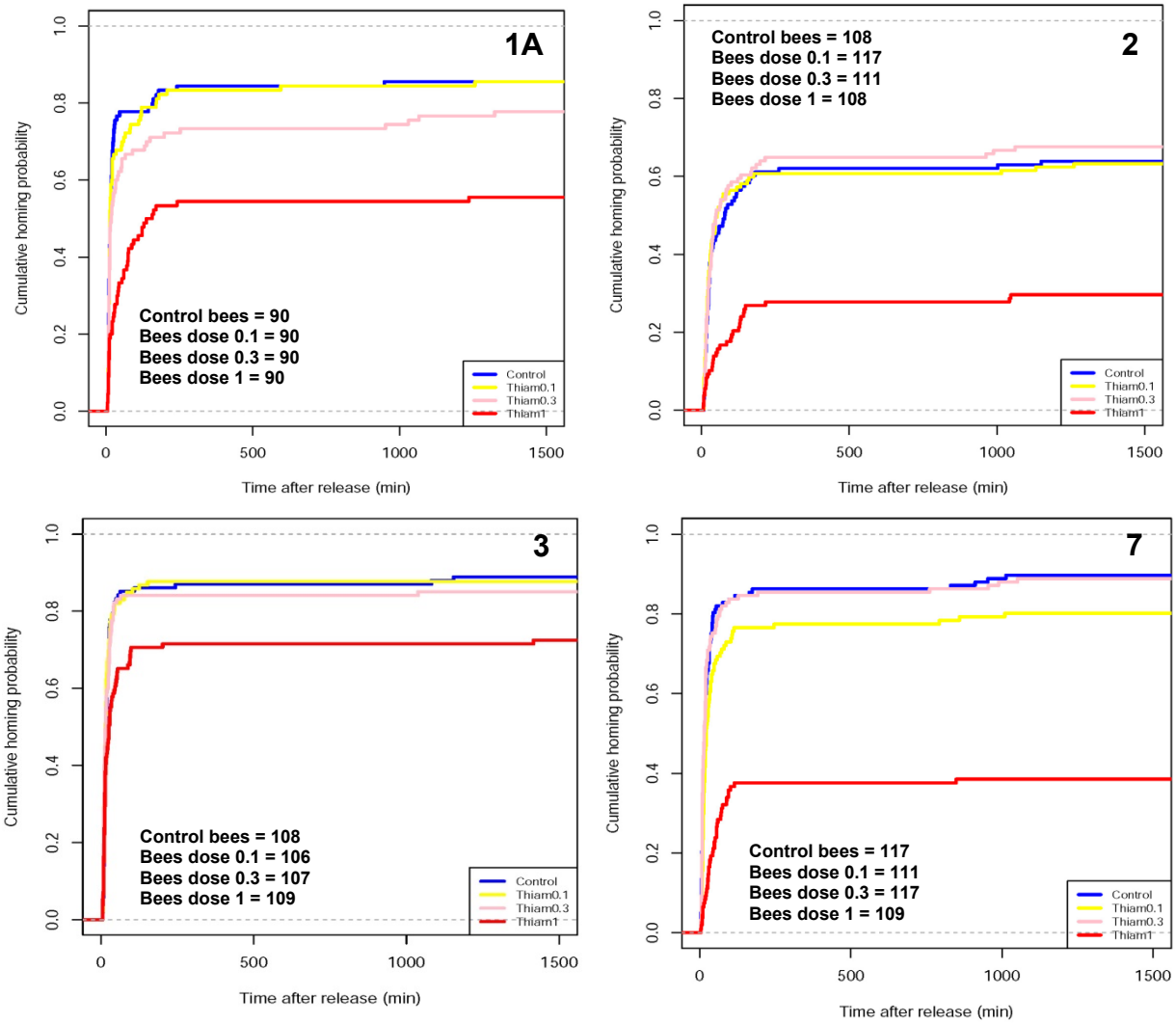


Figure 12: Cumulative homing probability of groups of foragers during 24 hours after release for the labs where a significant difference was found at least between control bees, bees exposed to 0.1-ng exposed bees and 1-ng exposed bees (3 test runs pooled, Chi² tests, $P < 0.05$). The yellow curve represents homing performances for foragers exposed to 0.11 ng per bee of thiamethoxam, the pink curve for the 0.33 ng per bee treatment, the red curve for the 1 ng per bee treatment and the blue curve for control bees.

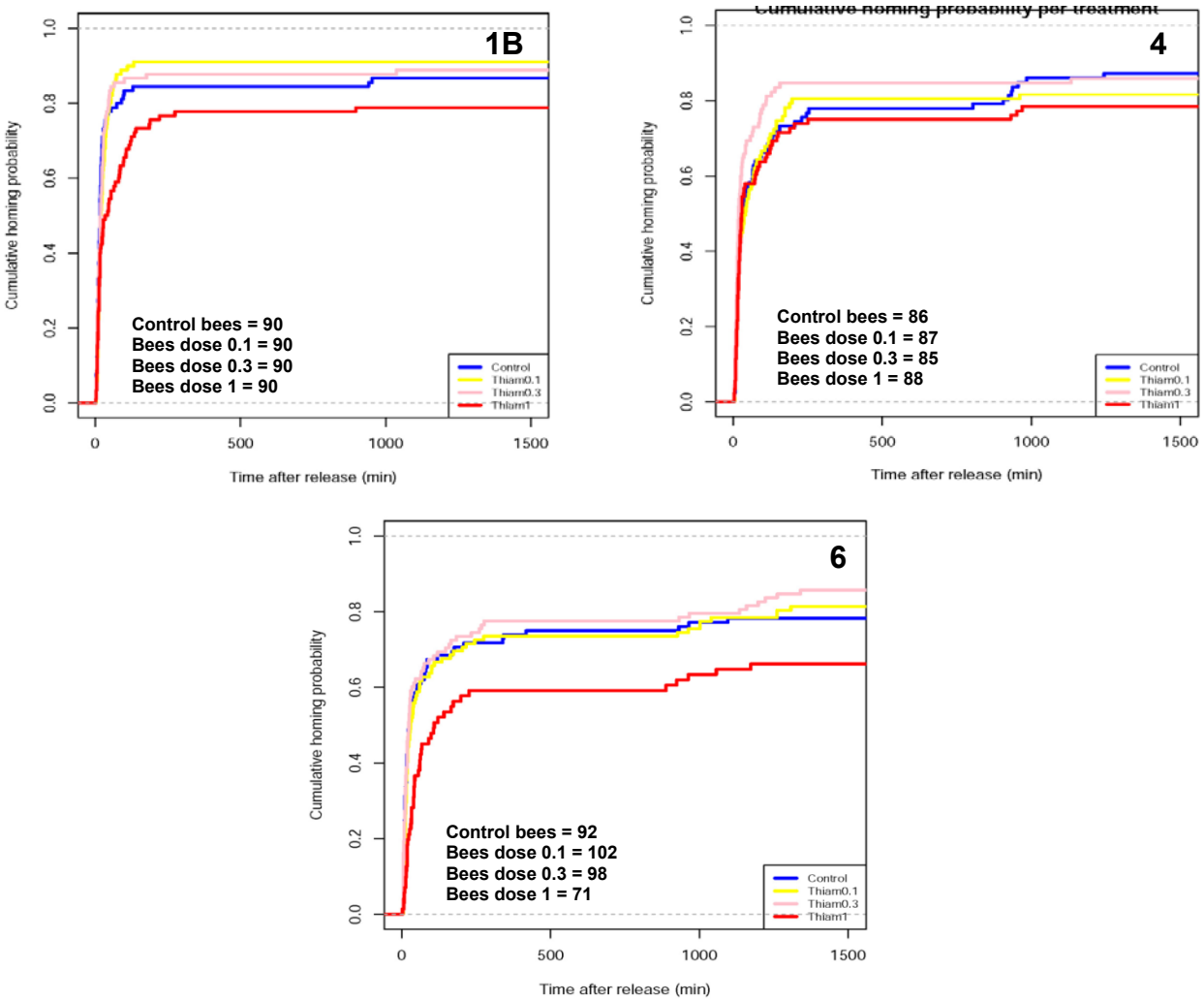


Figure 13. Cumulative homing probability of groups of foragers during 24 hours after release for the labs where no significant differences were found between the different groups of bees (3 test runs pooled, Chi^2 tests, $P > 0.05$). The yellow curve represents homing performances for foragers exposed to 0.11 ng per bee of thiamethoxam, the pink curve for the 0.33 ng per bee treatment, the red curve for the 1 ng per bee treatment and the blue curve for control bees.

Details of statistical analyses for homing performance of each laboratory are presented in **Appendix 9**.

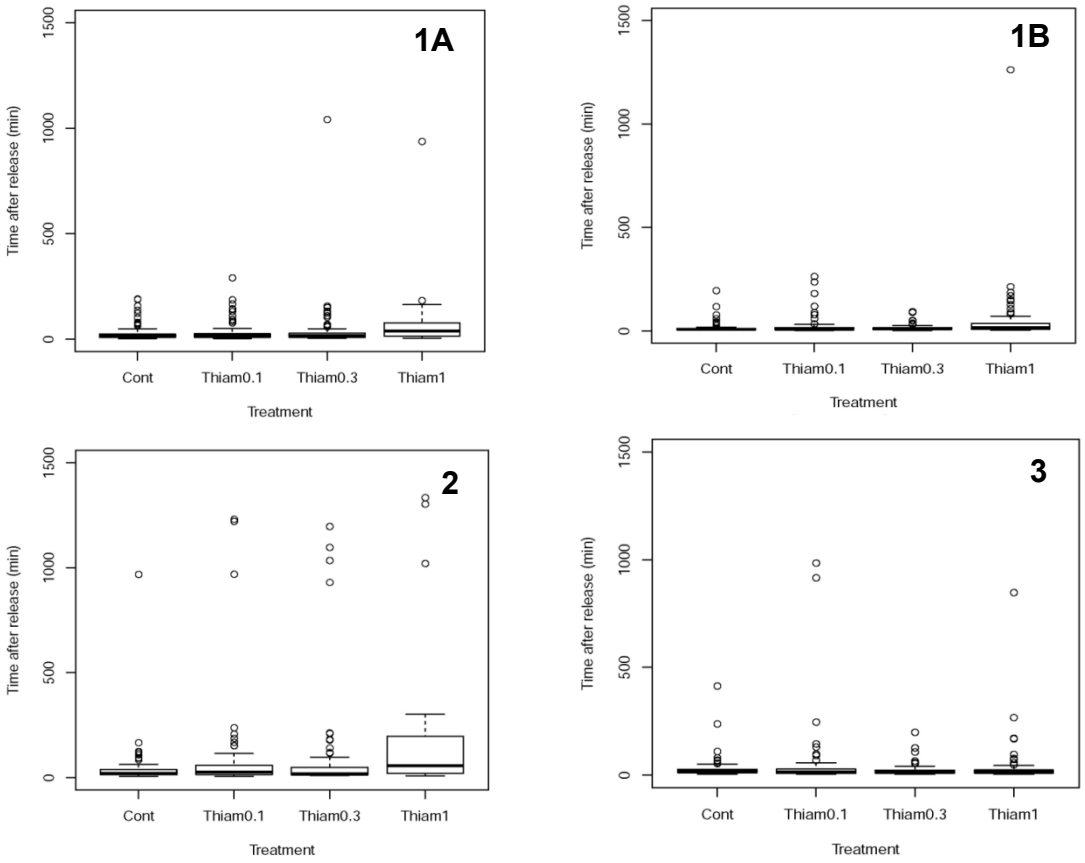
5.6 Homing duration per treatment

As a secondary observation, we calculated homing duration 24 hours after release.
In 2016, homing duration didn't significantly differ between groups of bees for 4 labs on 8 that performed valid tests (Kruskal-Wallis tests; $P > 0.05$; Table 15 and Figure 14). For 4 labs, homing duration was longer for bees exposed to the highest dose (Kruskal-Wallis tests followed by Mann-Whitney tests; $P < 0.05$; Table 15 and Figure 14).

Table 15: Median homing duration for the ring test 2016 (three valid test runs pooled)

		Control	Thiam. 0.11 ng/bee	Thiam. 0.33 ng/bee	Thiam. 1 ng/bee
Lab 1A	**Median homing duration in min (24 h after release)[1]**	**13.08 (a)**	**15.90 (a)**	**15.71 (a)**	**38.69 (b)**
Lab 1B		**8.53 (a)**	**8.54 (a)**	**9.53 (a)**	**14.91 (b)**
Lab 2		**20.68 (a)**	**25.45 (ab)**	**17.45(a)**	**55.98 (b)**
Lab 3		18.00 (a)	13.48 (a)	13.47 (a)	14.57 (a)
Lab 4		34.05 (a)	27.48 (a)	31.66 (a)	24.32 (a)
Lab 5		58.17 (a)	49.65 (a)	45.62 (a)	59.82 (a)
Lab 7		19.00 (a)	17.75 (a)	18.62 (a)	26.03 (a)
Lab 9		**18.26 (a)**	**22.88 (ab)**	**17.08(ab)**	**46.57 (b)**

[1]For homing duration, Kruskal-Wallis tests were performed. When a significant difference was found ($P < 0.05$), pairwise comparisons were performed using Mann-Whitney tests and Bonferroni P value adjustment method. Same letters indicate no significant differences.



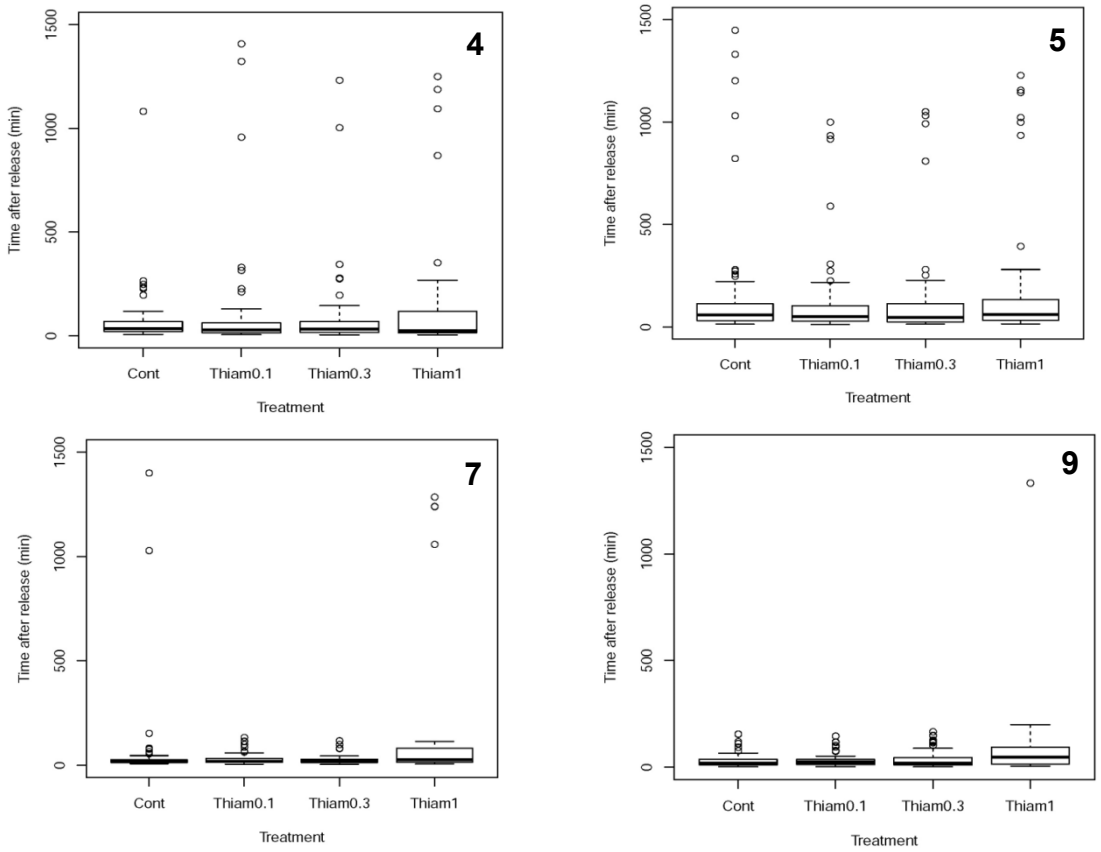


Figure 14: Homing duration of groups of foragers 24 hours after release in 2016 (three valid test runs pooled).

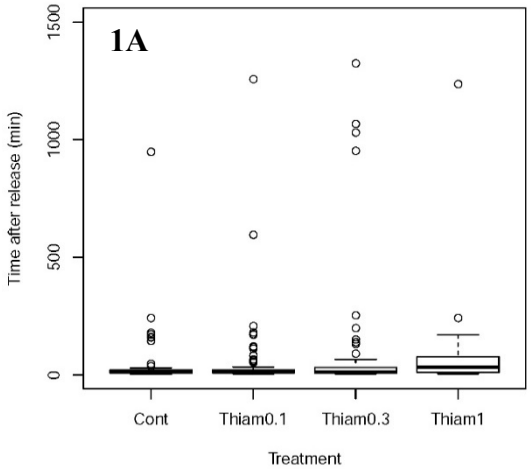
In 2017, homing duration didn't significantly differ between groups of bees for 4 labs on 7 (Kruskal-Wallis tests; $P > 0.05$; Table 16 and Figure 15). For 3 labs, homing duration was longer for bees exposed to the highest dose (Kruskal-Wallis tests followed by Mann-Whitney tests; $P < 0.05$; Table 16 and Figure 15).

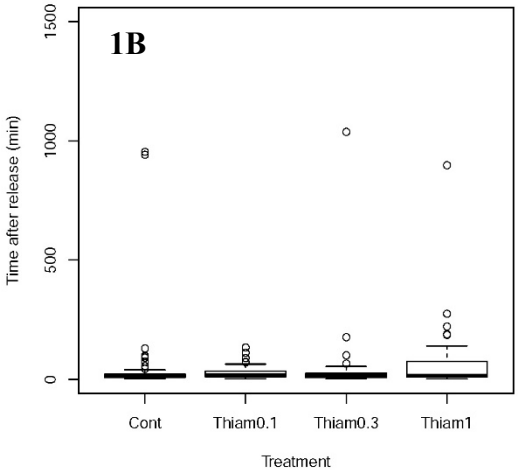
Table 16: Median homing duration for the ring test 2017 (three test runs pooled)

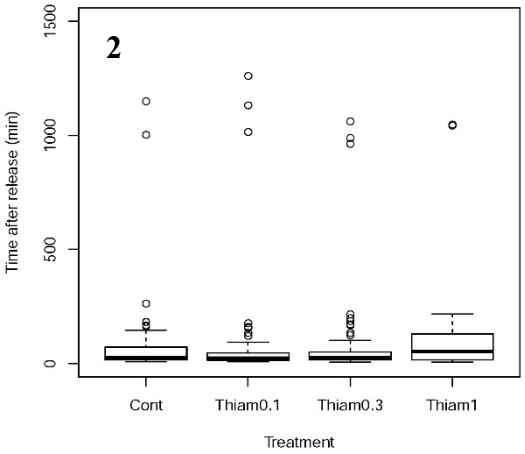
		Control	**Thiam. 0.11 ng/bee**	**Thiam. 0.33 ng/bee**	**Thiam. 1 ng/bee**
Lab 1A	**Median homing duration in min (24 h after release)[1]**	**12.12 (a)**	**12.32 (ab)**	**11.96 (ab)**	**33.44 (b)**
Lab 1B*		13.83 (a)	16.73 (a)	15.87 (a)	16.25 (a)
Lab 2		26.10 (a)	22.95 (a)	26.08 (a)	52.00 (a)
Lab 3		15.16 (a)	12.67 (a)	11.35 (a)	13.15 (a)
Lab 4		18.45 (a)	19.22 (a)	15.78 (a)	21.57 (a)
Lab 6		**16.23 (a)**	**18.98 (ab)**	**17.85 (a)**	**40.53 (b)**
Lab 7		**13.23 (a)**	**16.42 (a)**	**12.14 (a)**	**39.03 (b)**

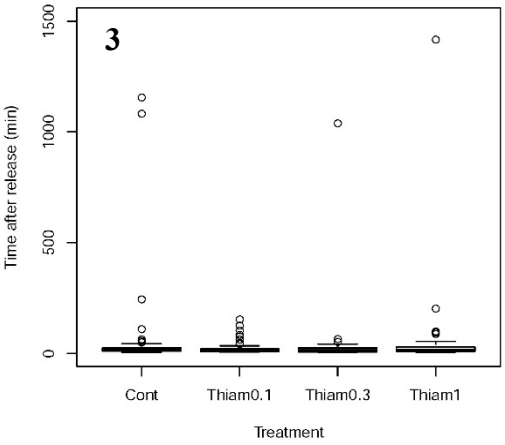
[1]For homing duration, Kruskal-Wallis tests were performed. When a significant difference was found ($P < 0.05$), pairwise comparisons were performed using Mann-Whitney tests and Bonferroni P value adjustment method. Same letters indicate no significant differences.

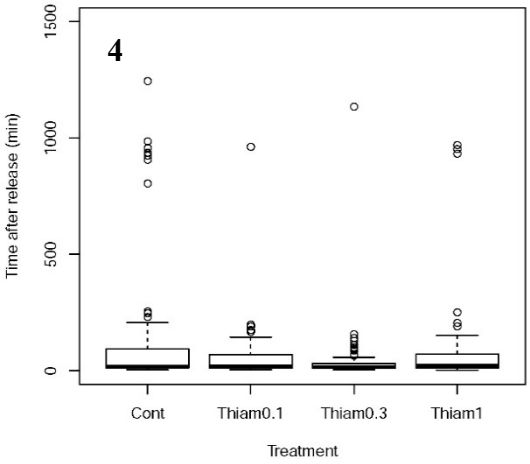
* 2 bees exposed per cage

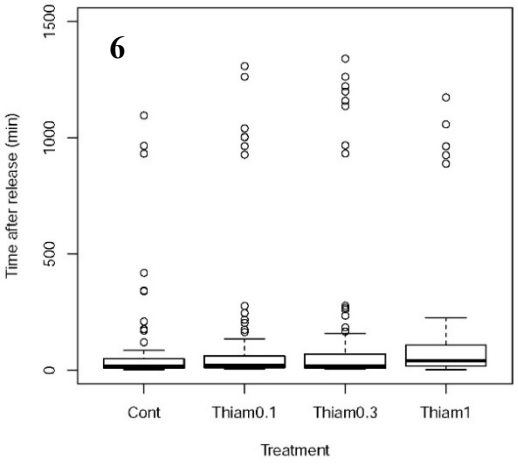

1A
Time after release (min)
0
500
1000
1500
Cont
Thiam0.1
Thiam0.3
Thiam1
Treatment


1B
Time after release (min)
0
500
1000
1500
Cont
Thiam0.1
Thiam0.3
Thiam1
Treatment


2
Time after release (min)
0
500
1000
1500
Cont
Thiam0.1
Thiam0.3
Thiam1
Treatment


3
Time after release (min)
0
500
1000
1500
Cont
Thiam0.1
Thiam0.3
Thiam1
Treatment


4
Time after release (min)
0
500
1000
1500
Cont
Thiam0.1
Thiam0.3
Thiam1
Treatment


6
Time after release (min)
0
500
1000
1500
Cont
Thiam0.1
Thiam0.3
Thiam1
Treatment

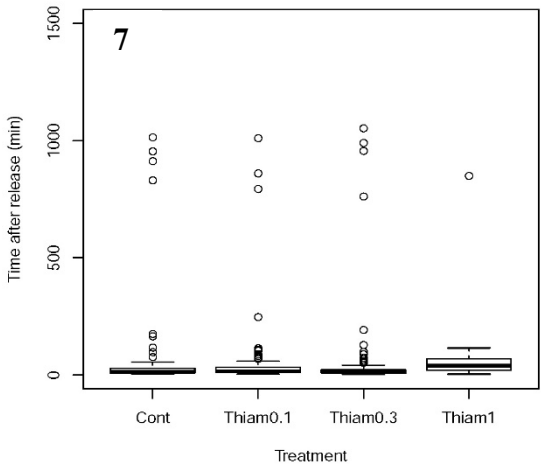


Figure 15: Homing duration of groups of foragers 24 hours after release in 2017 (three valid test runs pooled).

Details of statistical analyses for homing duration of each laboratory are presented in **Appendix 9**.

5.7 Analyses of variability of the homing performance

5.7.1 Variability of homing performance in control bees since 2015

For the majority of the tests runs, homing success rate ranged from the class [60-70[to the class [90-100] in 2015 and 2016, and progressed from the class [70-80[to the class [90-100] in 2017 (Table.17).

Table 17: Homing performance classes (%) in control bees for each test run of the labs

	2015		**2016**		**2017**	
Homing performance classes (%)	**Number of tests**		**Number of tests**		**Number of tests**	
[0-10[	0	**Nb of tests < to 60%* = 5**	0	**Nb of tests < to 60%* = 6**	0	**Nb of tests < to 60%* = 3**
[10-20[	0		1		0	
[20-30[	0		1		0	
[30-40[	2		0		0	
[40-50[	0		0		0	
[50-60[	3		4		3	
[60-70[	**7**	**Nb of tests > to 60%* = 17**	**5**	**Nb of tests > to 60%* = 25**	3	**Nb of tests > to 60%* = 22**
[70-80[	1		5		**4**	
[80-90[	3		5		11	
[90-100[	6		10		4	
Total test runs	**22**		**31**		**25**	

*Minimum and acceptable homing performance in control bees was considered at least of 60% for test validation based on results 2015

The median homing performance rate in control bees increases from 2015 to 2017 and were in the same range as results recorded in research and development studies performed at INRA Avignon and INRA Le Magneraud in 2012 and 2014 (Figure 16).

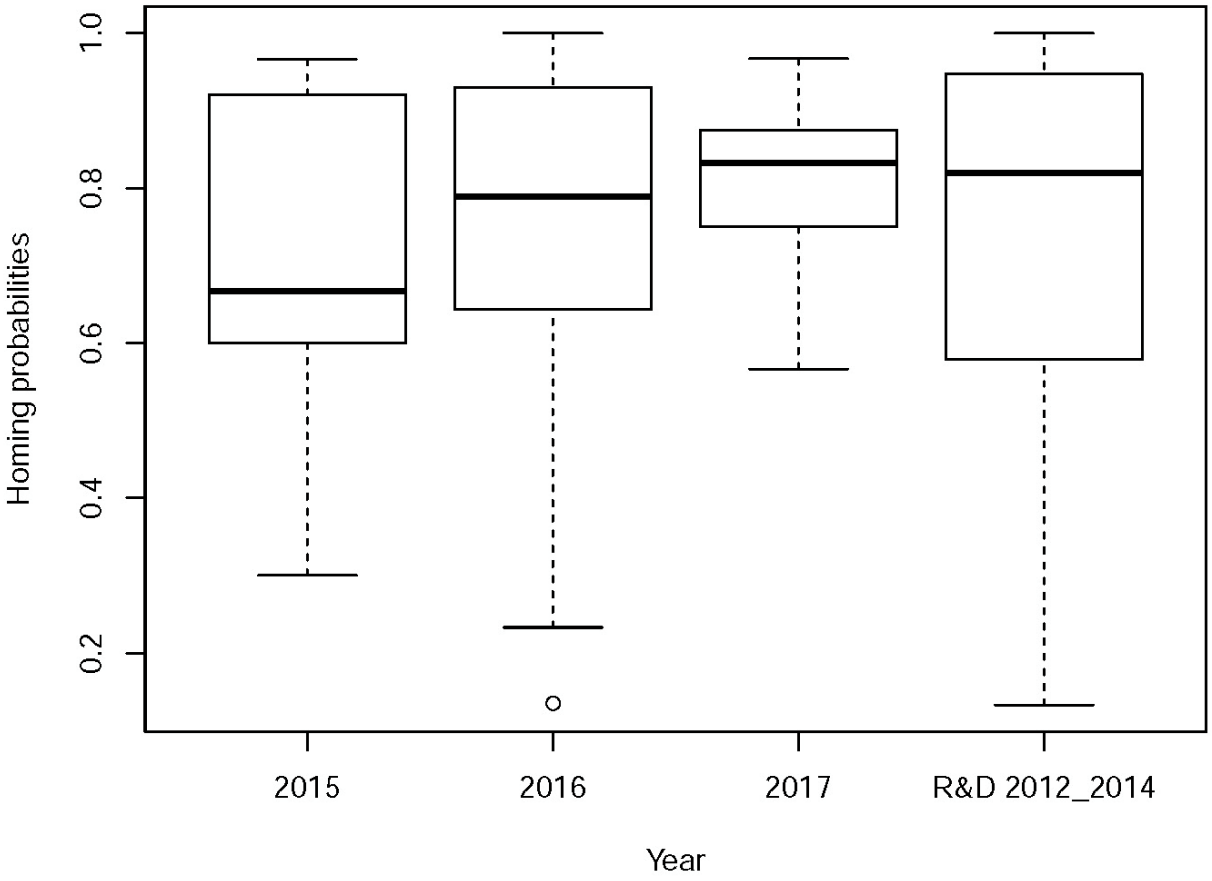


Figure 16: Median homing success probability (± SD) of control bees from 2015 to 2017 compared to results of research and development studies performed at INRA Avignon and INRA Le Magneraud in 2012 and 2014 ("R&D 2012_2014").

5.7.2 Analyses of the homing results variability

Homing results may be modulated by different factors like environmental conditions (e.g. climatic conditions, Henry et al. 2014) because homing performance is recorded in field conditions. Bioagressors and especially varroa infestation of the colonies might also have an effect (Monchanin et al. 2019). The effect of climatic conditions (mean temperature 24h-after release) on homing performance was assessed in 2016 and 2017. Varroa load of the colonies were also estimated for some labs in 2017. Real doses of test item were used for the analyses.

Results for all test runs (29 in 2016 and 20 in 2017) or for only valid ones (24 in 2016 and18 in 2017) were first considered according to the doses and temperatures (Tables 18 and 19). Generalized Linear Mixed Effects Models (GLMMs) showed no significant effect of temperature alone on homing performance of the bees in 2016. However, a significant effect of temperature in interaction with the dose was recorded by considering all the tests or only valid tests (Table 18, A and B). In 2017, statistical analyses showed a significant effect of temperature in interaction with the dose too but only when all the tests were considered (Table 19, A and B). Results showed that homing success of bees exposed to the highest dose increases with the temperatures.

Table 18. Summary of the generalized linear mixed effect models (GLMMs) performed in 2016 to assess the effect of the thiamethoxam dose, the mean temperature 24h-after release as well as their interactions on homing performance considering all the tests (A) or only valid tests (B).

A) All tests 2016 *

Model parameter	Averaged estimate ± s.e.	Z value	P value
Intercept	1.771 ± 0.553	3.200	< 0.01
Dose	-3.063 ± 0.281	-10.892	**< 0.0001**
Temperature	-0.427 ± 0.922	-0.463	0.643
Dose x Temperature	3.467 ± 0.628	5.522	**< 0.0001**

* Data of lab 8 were not included as real doses couldn't be analysed.

B) Valid tests 2016 *

Model parameter	Averaged estimate ± s.e.	Z value	P value
Intercept	2.343 ± 0.466	5.032	< 0.0001
Dose	-3.053 ± 0.300	-10.185	**< 0.0001**
Temperature	-0.741 ± 0.820	-0.904	0.366
Dose x Temperature	3.520 ± 0.629	5.599	**< 0.0001**

* Data of lab 8 were not included as real doses couldn't be analysed.

Table 19. Summary of the generalized linear mixed effect models (GLMMs) performed in 2017 to assess the effect of the thiamethoxam dose, the mean temperature 24h-after release as well as their interactions on homing performance considering all the tests (A) or only valid tests (B).

A) All tests 2017 *

Model parameter	Averaged estimate ± s.e.	Z value	P value
Intercept	1.929 ± 0.354	5.450	< 0.0001
Dose	-4.028 ± 0.535	-7.527	**< 0.0001**
Temperature	-0.545 ± 0.542	-1.006	0.315
Dose x Temperature	2.053 ± 0.924	2.222	**< 0.05**

*Results of lab 1B were excluded from the analyses as the exposure of the bees differed (2 bees exposed per cage *vs* 10 bees exposed per cage for the ring test). Results of the test run 3 of lab 6 were not included as real doses couldn't be analysed.

B) Valid tests 2017 *

Model parameter	Averaged estimate ± s.e.	Z value	P value
Intercept	1.951 ± 0.362	5.386	< 0.0001
Dose	-3.926 ± 0.539	-7.291	**< 0.0001**
Temperature	-0.308 ± 0.563	-0.548	0.584
Dose x Temperature	1.027 ± 0.969	1.060	0.289

*Results of lab 1B were excluded from the analyses as the exposure of the bees differed (2 bees exposed per cage *vs* 10 bees exposed per cage for the ring test). Results of the test run 3 of lab 6 were not included as real doses couldn't be analysed.

Details of the GLMMs results are presented in **Appendix 10**.

In 2017, varroa infestation of the colonies was also assessed for possible effect on homing success of the bees. Five laboratories of the ring test sampled honeybees from a brood comb. Honeybees were washed with water and detergent (Dietemann et al., 2013) in order to count the phoretic mites and establish the number of varroas per 100 honeybees (Lee et al., 2010). Results are presented in Table 20.

Table 20. Assessment of varroa infestation of the colonies of the ring test laboratories

Lab	Run	Sample date	Number of varroas per sample	Number of bees per sample	Number of varroas per 100 bees
1A	1	02/06/2017	3	160,7	1,9
1A	2	02/06/2017	0	185,0	0,0
1A	3	02/06/2017	0	260,0	0,0
1B	1	14/08/2017	18	315,7	5,7
1B	2	14/08/2017	2	271,4	0,7
1B	3	14/08/2017	2	202,9	1,0
2	1	07/06/2017	0	198,6	0,0
2	2	07/06/2017	5	277,1	1,8
2	3	07/06/2017	22	370,0	5,9
3	1	08/08/2017	0	184,3	0,0
3	2	21/08/2017	6	187,1	3,2
3	3	21/08/2017	3	190,0	1,6
4	1	22/06/2017	0	278,6	0,0
4	2	22/06/2017	3	250,0	1,2
4	3	22/06/2017	16	814,3	2,0
6	1	22/05/2017	0	85,7	0,0
6	2	12/06/2017	0	71,4	0,0

Results for valid test runs (14) were considered according to the doses and varroa. Generalized Linear Mixed Effects Models (GLMMs) showed an effect of varroa alone but no significant effect of varroa in interaction with the doses was recorded (Table 21). As a whole, from the data available and experimental conditions in 2017, varroa infestation of the colonies didn't affect the homing performances of exposed bees.

Table 21. Summary of the generalized linear mixed effect models (GLMMs) performed to assess the effect of the thiamethoxam dose, varroa infestation of the colonies as well as their interactions on honeybee homing success*

Model parameter	Averaged estimate ± s.e.	Z value	P value
Intercept	1.495 ± 0.300	4.990	< 0.0001
Dose	-2.707 ± 0.374	-7.243	**< 0.0001**
Varroa	0.547 ± 0.247	2.211	**< 0.01**
Dose x Varroa	-0.050 ± 0.874	-0.057	0.955

*Results of lab 1B were excluded from the analyses as the exposure of the bees differed (2 bees exposed per cage *vs* 10 bees exposed per cage for the ring test). Results of the test run 3 of lab 6 were not included as real doses couldn't be analysed.

Details of GLMMs results are presented in **Appendix 10**.

5.7.3. Effect of a feeding period before release

- **Pre-tests to compare the effects of a feeding *vs* no feeding period before release**

Since 2016, homing flight ring test protocol included a feeding period *ad libitum* before release to maintain good conditions of the foragers before release. However, as higher variability was observed in homing results of exposed bees (Figures 5 and 12), the effects of a feeding period before release were investigated. In 2017, two pre-tests were performed at ITSAP and INRA Le Magneraud labs.

Procedure: two tests were performed in May and June 2017 (ITSAP) and one test was performed in June/July 2017 (INRA Le Magneraud). Three test runs were performed according to the homing flight ring test protocol (see material and methods section). We compared the effects of control vs 1ng-per bee exposure for bees fed *ad libitum* or not before release with candy (ITSAP and INRA Le Magneraud) or with sucrose solution 30% (w/v) (ITSAP).

A summary of the percentage of bee mortality before release is presented in Table 22.

Table 22: Percentage of mortality (%) before release per treatment and run for each lab when comparing a feeding or no feeding period before release*

Lab	Run	Control fed	Control not fed	Thiam. 1 ng/bee fed	Thiam. 1 ng/bee not fed
ITSAP (candy)	**1**	10	3.3	0	0
	2	13.3	10	10	10
	3	0	0	3.3	0
ITSAP (sucrose solution)	**1**	0	0	0	3.3
	2	3.3	0	0	0
	3	0	0	0	3.3
Magneraud (candy)	**1**	16.7	0	13.3	16.7
	2	6.7	0.0	0.0	10
	3	0.0	3.3	0.0	0.0

*Percentages are established on 30 labelled bees per treatment and run

A summary of the number of bees released is presented in the Table 23.

Table 23: Number of bees released per treatment and run for each lab when comparing a feeding or no feeding period before release

Lab	Run	Control fed	Control not fed	Thiam. 1 ng/bee fed	Thiam. 1 ng/bee not fed
ITSAP (candy)	**1**	26	28	29	28
	2	23	27	24	23
	3	29	29	29	30
ITSAP (sucrose solution)	**1**	30	30	30	29
	2	29	28	28	29
	3	29	30	30	29
Magneraud (candy)	**1**	24	28	26	23
	2	28	30	28	26
	3	29	28	29	28

For ITSAP experiments, when bees are fed *ad libitum* before release (candi or sucrose solution), homing results of control bees and 1ng-exposed bees were comparable as a whole (Table 24). When the bees are not fed before release, lower homing performance was recorded for the 1ng-exposed bees compared to control bees. We also note no differences in homing success of control bees fed or not before release (Table 24).

For INRA Le Magneraud experiments, when bees are fed *ad libitum* before release (candi), homing results of the 1ng-exposed bees was lower that for control bees. Feeding before release improved homing performances especially in control bees, but lower results were obtained at this site as a whole (Table 24). Then, from one geographical site to another, results may differ when bees are fed before release.

Table 24: Results of homing probabilities per test run and lab when comparing a feeding or no feeding period before release

Lab	Run	Control fed	Control not fed	Thiam. 1 ng/bee fed	Thiam. 1 ng/bee not fed
ITSAP (candy)	**1**	0.692	0.750	0.690	0.500
	2	0.783	0.852	0.583	0.261
	3	0.793	0.690	0.828	0.467
ITSAP (sucrose solution)	**1**	0.600	0.800	0.700	0.414
	2	0.828	0.857	0.821	0.690
	3	0.793	0.900	0.667	0.793
Magneraud (candy)	**1**	0.750	0.036	0.038	0.043
	2	0.571	0.267	0.214	0.038
	3	0.724	0.535	0.414	0.393

Runs with no or low differences in homing success between 1ng-exposed bees and control bees (≤ 10 % of homing success between control and 1ng-bees) or homing success of 1ng-bees more important than control bees.

For ITSAP experiments and when the results of the 3 test runs are pooled, a significant difference is observed between control and 1ng-exposed bees only when the bees are not fed before release (Chi^2 tests; $P < 0.05$; Table 25). No significant differences are recorded when bees are fed with candy or sucrose solution 30% (w/v) before release. For Le Magneraud experiments, a significant difference is observed between control bees and the 1ng-exposed bees when the bees are fed before release (Chi^2 tests; $P < 0.05$; Table 25) but not when the bees are not fed as low results were obtained for both control and exposed bees.

Table 25: Homing performances 24 hours after release when the bees are fed *ad libitum* or not before release. Results of the 3 test runs were pooled.

		Control fed	Control not fed	Thiam. 1 ng/bee fed	Thiam. 1 ng/bee not fed
ITSAP (candy)	Number of foragers released	78	84	82	81
	Homing success probability (24 h after release) [1]	**0.756 (a)**	**0.762 (a)**	**0.707(a)**	**0.420 (b)**
ITSAP (sucrose solution)	Number of foragers released	88	88	88	87
	Homing success probability (24 h after release) [1]	**0.739 (ab)**	**0.852 (a)**	**0.727 (ab)**	**0.632 (b)**
Magneraud (candy)	Number of foragers released	81	86	83	77
	Homing success probability (24 h after release) [1]	**0.679 (a)**	**0.291 (b)**	**0.229 (b)**	**0.169 (b)**

[1]Pairwise comparisons were performed with Chi^2 test and used Bonferroni P value adjustment method. Same letters indicate no significant differences.

Details of statistical analyses for homing performances for each laboratory are presented in **Appendix 11**.

- **Research and development study to compare satiety level of the bees from ITSAP and INRA Le Magneraud labs**

One hypothesis of the difference in homing performances of control and exposed foragers fed or not before release, is the influence of the satiety level of the bees. Then, we weighted the crop content (mg) in sucrose solution of control and 1 ng-exposed foragers fed or not before release at the sites of INRA Le Magneraud and ITSAP. For this study, bees were fed only with 10 µl/bee of sucrose solution 30 % (w/v) before release (feeding treatment). We measured variable crop content especially when the bees were not fed before release. For Le Magneraud experiments, we measured low crop content when bees were not fed before release. Feeding the bees increased significantly crop content in both treated and control groups (Kruskal-Wallis test, P<0.0001; Figure 17). For ITSAP experiments, we measured relatively high resting crop content when the bees were not fed before release. Feeding the bees slightly increased crop content but there were no significant differences as a whole (Kruskal-Wallis test, P =0.051; Figure 17).

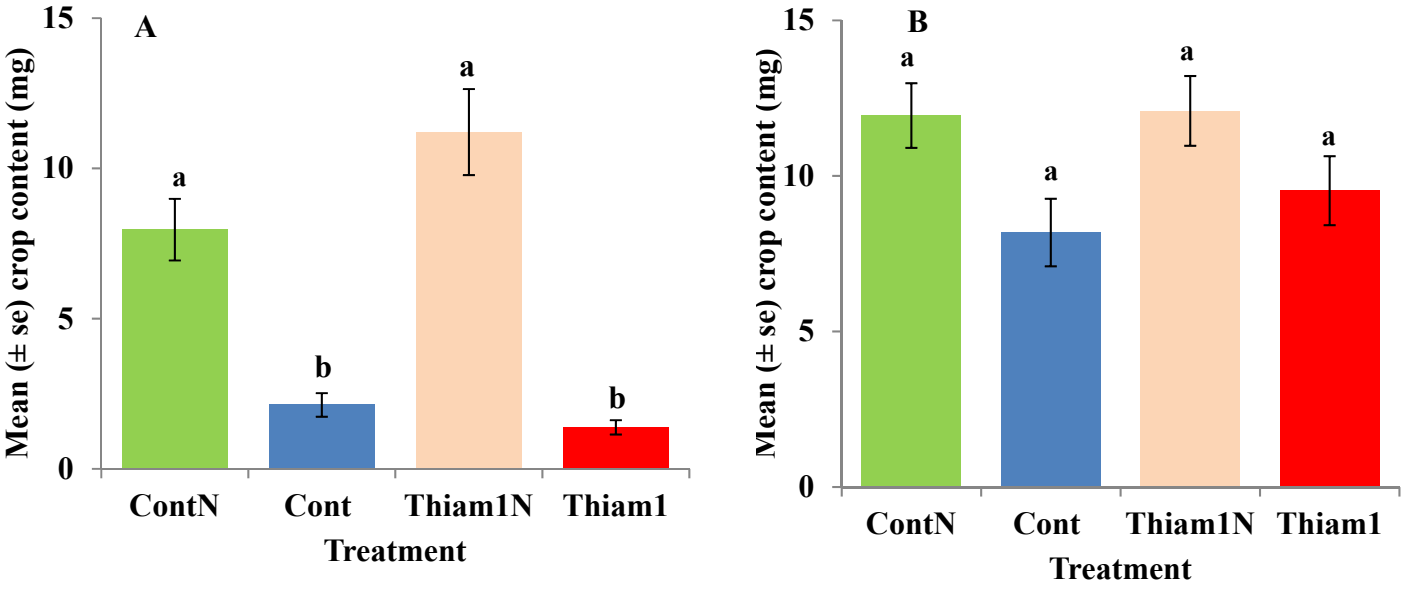


Figure 17. Mean (± standard error) of crop content in mg of control bees (Cont) and bees exposed to 1 ng of thiamethoxam (Thiam1) before release for **A**) INRA Le Magneraud lab **B**) ITSAP lab. N= bees fed before release with 10 µl / bee of sucrose solution 30% (w/v). Kruskal-Wallis tests were performed followed by Mann-Whitney tests and Bonferroni P value adjustment method when a significant difference was found (P < 0.05). Same letters indicate no significant differences.

In Avignon conditions, feeding the bees before release increases the crop content and the effects of the insecticide on homing performance decrease with the dilution of the remaining volume. This is especially true when bees are fed *ad libitum*. Environmental conditions as temperatures, resources available as well as bee genetic could explain homing results variability between the bees of ITSAP Avignon and INRA Le Magneraud.

Details of statistical analyses for crop content comparisons are presented in **Appendix 12**.

CONCLUSION

In the main, results showed the sensitivity of the homing flight method to detect the effect of low sublethal doses on homing success of foragers, with a common no observed effect dose (NOED) determined at 0.33 ng per bee. The powder method used as an alternative to the planting of a Phacelia field (ring test 2015) to ensure that the bees have a prior knowledge of the pathway back to the colony was easier to perform and showed its interest. In 2016, all the labs could perform the test compared to 2015. The powder used also showed no toxic effect alone or in interaction with the test item at the tested doses (see Appendix 6). Mean homing success in control bees increased from 2015 to 2017 going to a minimum and acceptable rate of 60 to 70 % for each test run performed.

Variability in homing results is also recorded especially for bees exposed to the highest dose. Temperature showed to have an effect on homing success of exposed bees. The methodological point consisting in feeding the bees *ad libitum* or with a limited amount before release is also an important factor of the results variability. As a whole, when bees are fed after the 1h-exposure starvation and before release, the crop content of the bees increases and the effects of the insecticide may decrease with the dilution of the remaining volume.

Consequently, the protocol was adjusted for the ring test 2018. The feeding period before release was suppressed but conditions of the bees during the laboratory phase and before release were optimized thanks to: the 1h-period of feeding *ad libitum* in the lab to synchronize the dietary state before the pre-exposure starvation, a pre-exposure starvation of 1h30 maximum compared to 2h in 2015 and 2016 and the decrease of the post-exposure starvation period from 1 h to 40 min as performed in the study of Henry et al. (2012).

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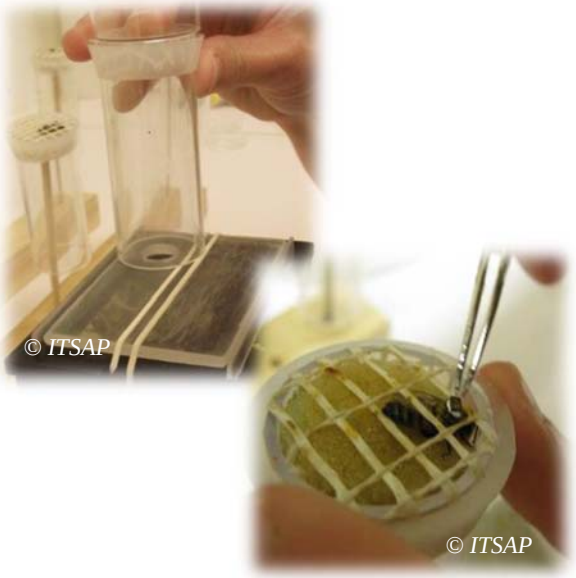
APPENDIX 1

Capture of the pink coloured bees at the hive entrance after release



Transfer in an holding cage and labelling with a RFID tag

Collective exposure phase

Example of a RFID system for the homing flight ring test

APPENDIX 2

Protocol to control performance of the RFID system

- 6 « test » tags glued onto small plastic or wooden sticks ➔ UIDs of the tags first recorded
- Each tag is passed five times through each of the four readers ➔ 20 readings per tag expected and a total of 120 readings expected for the 6 test tags
- Tested tag must be read at least one time each time it passes through a reader
- Reading rates (%) is calculated as recorded data on expected data (120 readings)

The acceptance criteria for the performance of the RFIS system was that **at least 95% of the crossing of the tags should be recorded**.

Reading rate control of the RFID system 2016

Laboratory	Total number of reading	Reading rate (%)
Lab 1	120	
Lab 2	120	98.3 %
Lab 3	120	98.3 %
Lab 4	120	100 %
Lab 5	120	98.3 to 99.17 %
Lab 6	120	100%
Lab 7	120	99.17 to 100 %
Lab 8	120	96.67 %
Lab 9	120	97.50 %
Lab 10	120	100%

Reading rate control of the RFID system 2017

Laboratory	Total number of reading	Reading rate (%)
Lab 1	120	
Lab 2	120	98.3 %
Lab 3	120	100 %
Lab 4	120	100 %
Lab 5	120	97.5 %
Lab 6	120	100 %
Lab 7	120	100 %
Lab 8	120	100 %

APPENDIX 3

A) 2016

Certificate of Analysis

Dr. Ehrenstorfer

Reference Materials for Residue Analysis

Product Identification

17453000 Thiamethoxam

CA 4H-1,3,5-Oxadiazin-4-imine, 3-[(2-chloro-5-thiazolyl)methyl]tetrahydro-5-methyl-N-nitro-

IUPAC 3-(2-Chloro-1,3-thiazol-5-ylmethyl)-5-methyl-1,3,5-oxadiazinan-4-ylidene(nitro)amine

Formula C8H10ClN5O3S

Mol.Weight 291.71

CAS No. 153719-23-4

Expiry Date 19.11.2017

Lot Number 31119

Store at 20 °C ±4 °C

Please note: The expiry date is valid under recommended storage conditions only.

Toxicological Data

R Code 22-53

S Code 60/61

LD50 (Rats female/male in mg/kg) 1563

Physical Data

Phase crystalline solid

Color colourless

Melt.Range 138.9 °C

Vapour pressure 6.6E-6 mPa at 25 °C

Solubility in water 4.1 g/l at 25 °C

Boiling Range (lit.)

Analytical Data

Detection: HPLC/DAD

Column: ReproSil 100 C18 5µ 250x3

Inj.-Vol.: 10.00 µl

Flow: 1.0 ml/min

Ret.-Time: 1.04 min.

Method Details:

Acetonitrile:H2O 4:1

Identity: UV, RT

Comment Purity was determined by external standard method.

Water Content 0.0 % Determined by Karl-Fischer Titration

Det. Purity 99.0 % Tolerance/Uncertainty +/- 0.5 %

The uncertainty/tolerance of this standard is calculated in accordance with the EURACHEM/CITAC Guide - Quantifying Uncertainty in Analytical Measurement - Second Edition. The uncertainty given is the expanded combined uncertainty and represents an estimated standard deviation equal to the positive square root of the total variance of the uncertainty of components. The expanded uncertainty is U which is Uc(y)*K, where K is the coverage factor at the 95% confidence level (K=2). The expanded uncertainty is based on the combination of uncertainties associated with each individual operation involved in the preparation of this product.

Certified on 15.01.2014

by A. Storr

The Laboratory Labor Dr. Ehrenstorfer-Schäfers is accreditated by DAkkS as indicated by the Accreditation Number D-RM-14174-01 has shown competence based on ISO Guide 34:2009 with relevant parts of DIN EN ISO/IEC 17025:2005 for production of certified reference materials in form of organic pure substances and in form of single and multi-component solutions organic pure substances.

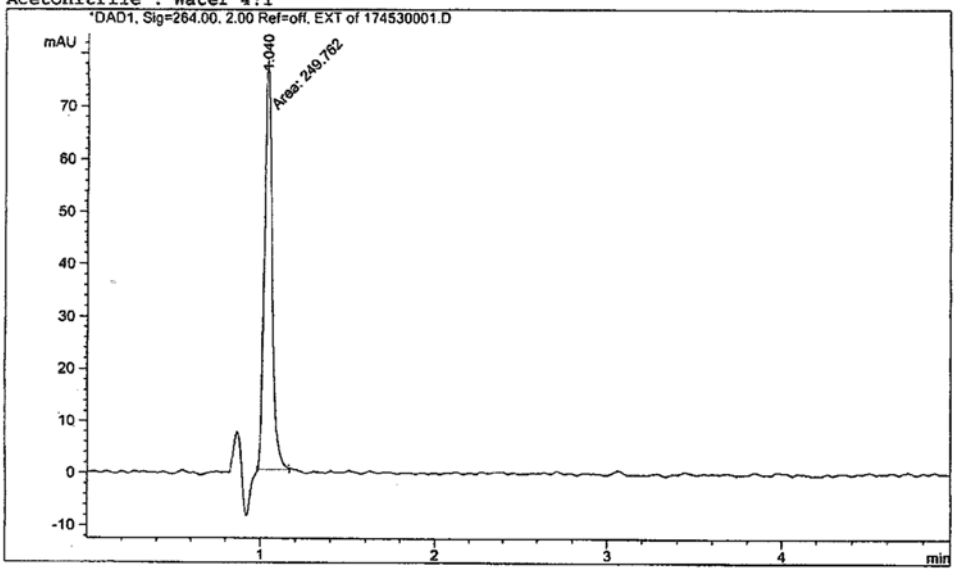
Labor Dr. Ehrenstorfer-Schäfers · Bgm.-Schlosser-Str. 6 A · 86199 Augsburg · Germany
Phone +49 821 906080 · Fax +49 821 9060888 · info@analytical-standards.com

Data File D:\CHEM32\1\DATA\201401KW02\174530001.D
Sample Name: 40109AL 31119

Thiamethoxam

Injection Date	: 11.01.2014 01:47:43	Seq. Line :	44
Sample Name	: 40109AL 31119	Location :	Vial 56
Acq. Operator	: DAD1_Admin	Inj :	1
Acq. Instrument	: Instrument 1	Inj Volume :	10 µl
Method	: D:\CHEM32\1\METHODS\41K.M		
Last changed	: 05.12.2013 10:04:25 by DAD1_Admin		

Acetonitrile : Water 4:1



Area Percent Report

Sorted By : Retention Time
Multiplier : 1.0000
Dilution : 1.0000
Use Multiplier & Dilution Factor with ISTDs

Signal 1: DAD1, Sig=264.00, 2.00 Ref=off, EXT
Signal has been modified after loading from rawdata file!

Peak #	RetTime [min]	Sig	Type	Area [mAU*s]	Height [mAU]	Area %
1	1.040	1	MM T	249.76204	78.99778	100.0000
Totals :				249.76204	78.99778	

*** End of Report ***

B) 2017

FOURNISSEUR HPLC & GC DEPUIS 1981
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email : cil@cluzeau.com
web : www.cluzeau.com
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33220 SAINTE-FOY-LA-GRANDE
☎ : 05 57 46 00 44

Dr. Ehrenstorfer
Reference Materials for Residue Analysis

Certificate of Analysis

ISO Guide 34 Reference Material
Product Identification

Articel Code:	DRE-C17453000	**Lot Number:**	G119775
Artikel Name:	Thiamethoxam	**Expiry Date:**	16.01.2021
Formula:	C8H10ClN5O3S	**Storage Temperature:**	20°C ± 4°C
Mol. Weight:	291.71		
CAS No.:	153719-23-4		

Storage and handling: The RM should be stored in the original sealed bottle at the temperatur given above. After use the bottle should be tightly closed and protected from moisture and light. The expiry date is valid for original closed bottles under recommended storage conditions only.

Purity	99.69%
Expanded Uncertainty U=	0.30%

The uncertainty of this standard is calculated in accordance with the ISO Guide 34 and EURACHEM/CITAC Guide - Quantifying Uncertainty in Analytical Measurement, Second Edition. The Expanded uncertainty is U = u(RM) x k, where k i the coverage factor at the 95% confidence Level (k=2). The expanded uncertainty U is bades on the combination of the uncertainties associated with each individual operation involved in the analysis of the product. $U(RM) = \sqrt{u(char)^2 + u(bb)^2 + u(lts)^2 + u(sts)^2}$; u(char) is the uncertainty of purity determination; u(bb) uncertainty of homogeneity test; u(lts) is uncertainty of stability test long-term; u(sts) is uncertainty of stability test short-term. u(lts) and u(sts) are not included in the calculation as the stability statement is based on real evidence opposed to simulation.
Minimum sample: 1 mg is recommended as the minimal sample amount. If less material is used, it is recommended to increase the certified uncertainty by a factor of two for half sample and a factor of four for a quarter of sample
Intended use: Use this RM as calibrant for chromatography or any other analytical technique.

Analytical Data

Traceability of chromatography: To the International System of Unity (SI).

Intrument:	HPLC/DAD	Method Details
Detection:	DAD	Acetonitrile:Water 4:1
Column:	ReproSil 100 C18 5 µm 250 x 3 mm	
Inj.-Vol.:	10 µl	
Flow:	1 ml/min	
Ret.Time:	1.15 min	

Comment
Traceability: The balances used are calibrated with weights traceable to the national standards (DKD).
Calibrated Class A glassware is used for volumetric measurements.
Certificate Revision 1
Water Content: 0.26% (g/g) by Karl-Fischer-Titration (U(exp) = 0.22% (g/g)).
Identity: EA, NMR, RT, IR, UV

Certified on: 16.01.2017
Certified by: M. Beck

M. Beck

Authorized copy
from the original
18. JAN. 2017
Sign..

The Laboratory LGC Labor GmbH is accreditated by DAkkS as indicated by the Accreditations Number D-RM-19883-01 & D-PL-19883-01 has shown competence based on ISO Guide 34:2009 with relevant parts of DIN EN ISO/IEC 17025:2005 for production of certified reference materials in form of organic pure substances and in form of single and multi-component solutions of organic pure substances.

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13.01.17 HQ

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		Inj Volume	: 10.000 µl

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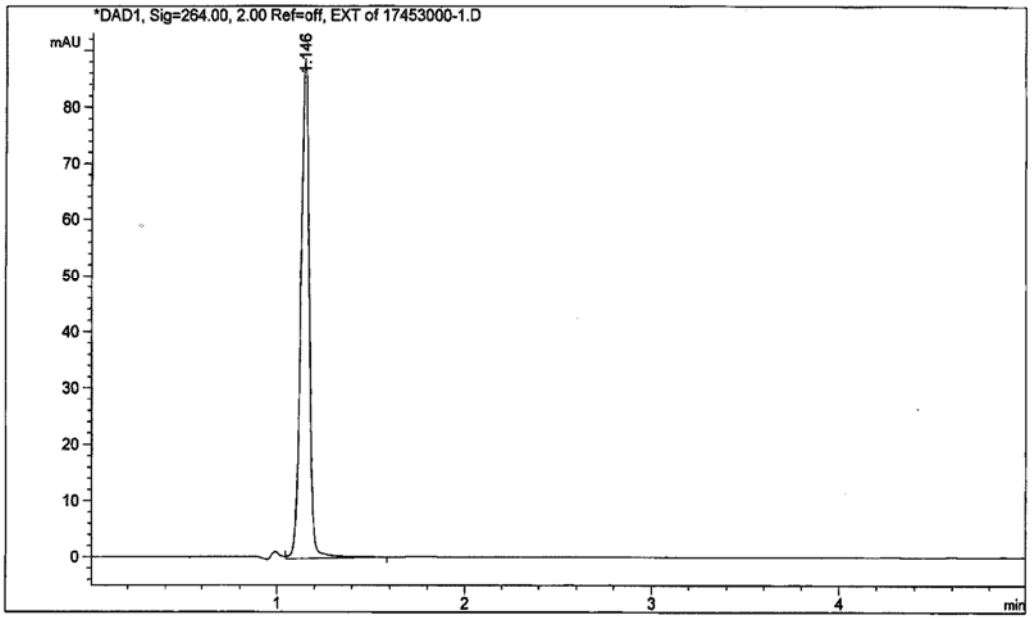
Sample Info : Thiamethoxam



Area Percent Report

Sorted By : Retention Time
Multiplier : 1.0000
Dilution : 1.0000
Use Multiplier & Dilution Factor with ISTDs

Signal 1: DAD1, Sig=264.00, 2.00 Ref=off, EXT
Signal has been modified after loading from rawdata file!

Peak #	RetTime [min]	Sig	Type	Area [mAU*s]	Height [mAU]	Area %
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Totals :				280.75775	89.04220	

*** End of Report ***

LCMS 13.01.2017 10:01:14 SYSTEM Page 1 of 1

APPENDIX 4

Preparation of the item, test solutions and test feeding solutions

- ⇨ 20 µl of sucrose solution (30 % w/v) per bee containing 0.1% of acetone is considered
- ⇨ Test item doses: 0.11, 0.33 and 1 ng per bee

1- Preparation of the stock solution (S)

**1 ng test item in 0.02 µl acetone => 50 ng/µl or 50 µg/ml*

Preparation of a one hundred time more concentrated « S »:

50 x 100 = 5 000 µg/ml or 5 mg/ml

To prepare « S » => 10 mg of thiamethoxam is weighed and 2 ml of acetone is added

2- Preparation of a 1ng per bee test solution (S1)

Dilution 1/100: solution « S1 » at 50 µg/ml

10 ml as a final acetone volume is considered.

Preparation:

Ci xVi = Cf xVf => 5000 µg/ml x Vi = 50 µg/ml x 10

Vi = 0,1 ml => 100 µl of S is sampled and 9.9 ml of acetone is added

3- Preparation of a 0.33 ng per bee test solution (S2)

Dilution 1/3: solution S2 at 16.667 µg/ml

Preparation : 1 ml of S1 is sampled and 2 ml of acetone is added

4- Preparation of a 0.11 ng per bee test solution (S3)

Dilution 1/3: solution S3 at 5.556 µg/ml

Preparation : 1 ml of S2 is sampled and 2 ml of acetone is added

5- Test feeding solutions

General preparation: 15 g of sugar in 50 ml of demineralized water (30 % w/v)

Four samples of 10 ml of this sucrose solution are prepared for the 3 tested and control treatments.

Test feeding solutions are prepared in 10 ml of sucrose solution:

Treatment	Test solution sample in µl	Sucrose solution (30% w/v) in ml
Control (acetone)	10 µl acetone	10
Thiamethoxam 1 ng	10 µl S1	10
Thiamethoxam 0.33 ng	10 µl S2	10
Thiamethoxam 0.11 ng	10 µl S3	10

APPENDIX 5

As in 2015, pre-tests were performed in 2016 to assess the effect of acetone solvent on homing success when used as a test control.
Two tests with two runs per test were performed in May (Test 1, ITSAP lab, Lyon) and three runs per test in June/July (Test 2, INRA Le Magneraud lab) 2016. Protocol applied for capture, labelling and exposure, release… was the same as the homing flight ring test protocol with methodological points applied in 2016 (see material and method section).We tested 3 different volumes of acetone including the highest acceptable volume for an homing test (0.1 % (v/v), 0.5 % (v/v) and 1 % (v/v) of sucrose solution 30 % (w/v)) and we compared to a water control (1% (v/v) of sucrose solution 30 % (w/v)).

Results of homing probabilities per test run and lab are presented in Table 1 Appendix 5.The different tested acetone volume didn't show any effect on homing performances. For all the acetone treatments tested, bees returned to the hive in the same proportions than bees exposed to water in the feeding solution (Chi^2 tests, $P > 0.05$; Table 2 Appendix 5). Bad weather conditions experienced in May during the Test 1 can explain low homing success obtained.

Table 1 Appendix 5: Results of homing probabilities per test run and laboratory

Lab	Run		Control	Thiam. 0.1ng/bee	Thiam. 0.3 ng/bee	Thiam. 1 ng/bee
ITSAP	**1**	Nb of released bees	28	27	28	28
		Homing probabilities	0.393	0.481	0.536	0.536
	2	Nb of released bees	30	30	30	30
		Homing probabilities	0.567	0.533	0.567	0.567
INRA	**1**	Nb of released bees	26	27	28	28
		Homing probabilities	0.885	0.852	0.821	0.786
	2	Nb of released bees	39	40	39	38
		Homing probabilities	0.692	0.800	0.718	0.526
	3	Nb of released bees	29	28	30	29
		Homing probabilities	0.483	0.643	0.667	0.724

Table 2 Appendix 5: Homing success results for acetone experiments (test runs pooled for each test)

A)Test 1: ITSAP	**Control**	**Acetone 0.1% v/v**	**Acetone 0.5% v/v**	**Acetone 1% v/v**
Number of foragers released	57	58	58	58
Homing success probability (24 h after release) [1]	0.483 (a)	0.509 (a)	0.552 (a)	0.552 (a)

B) Test 2: INRA Le Magneraud	**Control**	**Acetone 0.1% v/v**	**Acetone 0.5% v/v**	**Acetone 1% v/v**
Number of foragers released	95	97	95	94
Homing success probability (24 h after release) [1]	0.681 (a)	0.768 (a)	0.732 (a)	0.663 (a)

[1]Pairwise comparisons were performed with Chi² test and used Bonferroni P value adjustment method. Same letters indicate no significant differences.

Statistical analyses with R software version 3.3.1 (R Development Core Team, 2008) on homing results

Test 1: ITSAP

Pearson's Chi-squared test

data: tab_cont

X-squared = 0.80591, df = 3, p-value = 0.8481

Test 2: INRA Le Magneraud

Pearson's Chi-squared test

data: tab_cont

X-squared = 3.2075, df = 3, p-value = 0.3607

APPENDIX 6

Comparative acute oral toxicity in combination with and without hydrophobic powder to the honeybee under laboratory conditions

Background and objective

A pre-test was conducted in 2016 to assess the coloured hydrophobic pink powder (COLOREY SAS, France) oral toxicity alone or in interaction with the test item tested. A sub-group of the ring test group was volunteered to perform the pre-test. Powdered bees *vs* non-powdered bees were compared. The objective was to calculate a median lethal dose ($LD_{10,20,50}$) 24h and 48h-after exposure where possible. The test was conducted according to the test guideline OECD N°213 (1998).

Test procedure

1- Bees collection

Non-powdered bees: collection of the bees on outer combs before being introduced in test units (cages)

Powdered bees (**quantity tested : 0.5 mg per bee**):

Method 1 (Laboratory): collection of the bees on outer combs, powdering in plastic boxes in laboratory and introduction in test units thanks to a tube between boxes and test unit (cages) : powdered bees can walk from boxes to test units (they shake off of the excessive powder during transit)

Method 2 (Field): collection of the bees on outer combs, powdering in plastic boxes, release at 3 meters from the hive (to shake off of the excessive powder) and collection in tests units (cages) before being transported to laboratory

Moribund bees are replaced by healthy one before starting the test

2- Test design

Number of treatment groups	-2 control groups: powdered vs non-powdered -12 test item groups: T1-T6 for powdered bees, T1-T6 for non-powdered bees
Feeding solution during exposure phase	Sucrose solution 30 % (w/v) with 20 µl per bee
Replicates per treatment group	4
Number of bees	Ten bees per cage and 4 replicates (Total = 40 bees per treatment)

3- Treatment groups for powdered and non-powdered bees

-Control acetone (0.1% v/v)

-Treatment groups (T1 to T6): 0.11, 0.33, 1, 3, 9, 18 ng per bee in acetone 0.1% (v/v)

Results

Four laboratories of the ring test participated to this pre-test. Lab 4 could only assess the effects 24h after exposure. Individual data from each of the labs of presented in Table 1 Appendix 5 for non-powdered bees and powdered bees.

Table 1 Appendix 5: Cumulative mortality (%) in the control and the different test item treatments for non-powdered bees (A) and for powdered bees (B)

A)

Treatment	**24 h after exposure**				**48 h after exposure**			
	Lab1	Lab2	Lab3	Lab4	Lab1	Lab2	Lab3	Lab4
Control	0	2.5	0.0	0.0	2.5	2.5	2.5	-
0.11	2.5	2.5	0.0	0.0	2.5	2.5	0	-
0.33	0	0.0	2.5	0.0	2.5	2.5	2.5	-
1	2.5	0.0	0.0	0.0	5	2.5	0	-
3	7.5	15.0	35.0	22.5	10	15	35	-
9	37.5	80.0	62.5	85.0	37.5	80	65	-
18	55	92.5	77.5	95.0	57.5	95	77.5	-

B)

Treatment	**24 h after exposure**				**48 h after exposure**			
	Lab1	Lab2	Lab3	Lab4	Lab1	Lab2	Lab3	Lab4
Control	0.0	2.5	2.5	0.0	5	2.5	5	-
0.11	0.0	2.5	0.0	0.0	0	2.5	0	-
0.33	2.5	0.0	2.5	2.5	5	0	5	-
1	2.5	2.5	7.5	0.0	5	5	7.5	-
3	12.5	32.5	32.5	2.5	12.5	32.5	32.5	-
9	62.5	90.0	80.0	7.5	62.5	90	80	-
18	57.5	100.0	92.5	52.5	62.5	100	92.5	-

The doses which cause 10 (LD_{10}), 20 (LD_{20}) and 50 % (LD_{50}) mortality at 24 and 48 hours after insecticide exposure were determined using Benchmark Dose (BMDS) Software 2.6.0.1. Parsimonious models were based on the Akaikee information criterion (AIC).

Table 2 Appendix 5. Calculation of LD_{10}, LD_{20}, and LD_{50} at 24 and 48 hours after exposure to the insecticide.

A) Lab 1

		Powdered bees*	**Lower limit (95 % confidence limits)**	Non-powdered bees**	**Lower limit (95 % confidence limits)**
24 h	LD_{10} (ng/bee)	1.85	1.15	3.35	2.06
	LD_{20} (ng/bee)	3.46	2.48	5.58	3.99
	LD_{50} (ng/bee)	**10.12**	**7.90**	**14.83**	**11.48**
48 h	LD_{10} (ng/bee)	2.13	1.20	3.18	1.69
	LD_{20} (ng/bee)	3.74	2.50	5.37	3.51
	LD_{50} (ng/bee)	**9.81**	**7.68**	**14.62**	**11.19**

* LogLogistic model for powdered bees
** LogProbit model used for non-powdered bees

B) Lab 2

		Powdered bees*	**Lower limit (95 % confidence limits)**	Non-powdered bees**	**Lower limit (95 % confidence limits)**
24 h	LD_{10} (ng/bee)	1.79	1.27	2.61	1.93
	LD_{20} (ng/bee)	2.49	1.94	3.52	2.78
	LD_{50} (ng/bee)	**4.35**	**3.70**	**5.89**	**4.96**
48 h	LD_{10} (ng/bee)	1.65	1.15	2.65	1.91
	LD_{20} (ng/bee)	2.36	1.81	3.54	2.73
	LD_{50} (ng/bee)	**4.27**	**3.61**	**5.79**	**4.84**

* Gamma model used for powdered bees
** LogLogistic model used for non-powdered bees

C) Lab 3

		Powdered bees*	**Lower limit (95 % confidence limits)**	Non-powdered bees**	**Lower limit (95 % confidence limits)**
24 h	LD_{10} (ng/bee)	1.36	0.90	1.29	0.86
	LD_{20} (ng/bee)	2.11	1.54	2.22	1.63
	LD_{50} (ng/bee)	**4.46**	**3.61**	**6.30**	**5.03**
48 h	LD_{10} (ng/bee)	1.45	0.92	1.50	0.96
	LD_{20} (ng/bee)	2.22	1.57	2.47	1.75
	LD_{50} (ng/bee)	**4.60**	**3.69**	**6.38**	**5.10**

* LogLogistic model for powdered bees
** LogProbit model used for non-powdered bees

D) Lab 4

		Powdered bees*	**Lower limit (95 % confidence limits)**	Non-powdered bees**	**Lower limit (95 % confidence limits)**
24 h	LD_{10} (ng/bee)	10.10	8.52	2.14	1.61
	LD_{20} (ng/bee)	12.86	11.40	2.86	2.27
	LD_{50} (ng/bee)	**17.71**	**16.11**	**5.01**	**4.25**

* Logistic model for powdered bees
** LogProbit model used for non-powdered bees

Results of $LD_{10,20,50}$ varied from one lab to another and between groups of bees (« powdered » vs « non-powdered »). But for all the labs, the comparative acute toxicity study showed that the pink hydrophobic powder had no abnormal negative effects on bee mortality compared to bees not powdered at the tested doses, whether exposed or not to the insecticide compared to bees not powdered at the tested doses. The LD_{10} estimate was above our nominal tested doses (0.11, 0.33 and 1 ng per bee) for both powdered and non-powdered bees.
It has to be noted that for lab 4, $LD_{10,20,50}$ were higher for powdered bees than for non-powdered bees. Powdered bees consumed less sucrose solution during exposure phase because of cleaning activity.

APPENDIX 7

Pre-test of Lab 3 in 2016: Tag loss rate of the bees

Background

One hypothesis of the variability in homing results, especially homing success in control bees could be tag loss during the return flight of the foragers. The objective of this pre-test was to estimate how many foragers lose their tags during homing flight by estimating the ratio of incoming bee with tags *vs* incoming bees which lost their tags during the homing flight.

Test procedure

Procedure to collect the bees in field conditions was similar to the ring test protocol 2016.
From bees collected, 100 were used and labelled with a dummy tag. Additionally, the bees were colored with green "queen color" necessary to distinguish the incoming bees (green plus tag) from bees which lost their tag (only green).
After release of green and labelled bees, they were captured for one hour in a special box ("capture box") at the hive entrance. Bees flew in, but were not able to fly out. Shortly before the 2nd Release the capture box was closed with a lid which allowed contact but no access to the colony. After one hour, the capture box was removed and transported to the laboratory. The bees were anesthetized with CO2 and those with tag and green color were separated and counted.
One hour for collecting the bees was set by Lab 4 because most of the bees were back 30 minutes after release during the homing flight results 2015.

Results

It was noted only 1 tag loss shortly before release. Then, 99 bees were released. The homing success was 30.3 % (30 of 99 bees returned to the colony). The low homing success was probably due to the additional green color, which agglutinated the bees too much. Before release, we observed many bees unable to fly with agglutinated wings. The green color dried very slowly. Nonetheless, all of the 30 bees, captured in the box at the colony, still carried their tag. Then, the dental cement alone seems to be appropriate to label the foragers and to follow their homing flight.

APPENDIX 8

A) Punctual weather conditions (temperature, hygrometry, cloud layer and wind strength) at the time of the bees release for each lab and run during the homing flight ring test 2016

Lab	Run	Mean temperature (°C)	Mean hygrometry (%)	Cloud layer	Wind strength
1A	1	31.5	36	Average	Low
	2	33.5	39.5	Low	Low
	3	32	47	Average	Low
1B	1	28.5	36	Null	Low
	2	24.7	39.5	Low	Low
	3	26.2	38.5	Average	Average
2	1	24.6	54.2	Low	Low
	2	27.4	40.9	Average	Null
	3	26.2	53	Average	Null
3	1	25	37	Null	Null
	2	25	35	Average	Low
	3	31	34	Null	Low
4	1	36	18	Null	Null
	2	33.7	32	Null	Low
	3	32.7	27	Null	Low
5	1	26.4	59	Low	Low
	2	31.7	57	Null	Average
	3	21.9	45	Average	Low
6	1	21.5	79.5	Null	Low
	2	23.5	74.5	Null	Low
	3	29	50	Null	Low
7	1	-	-	High	Low
	2	-	-	Average	Low
	3	-	-	Null	Low
8	1	31	42	Low	Null
	2	27.8	51	Null	Null
9	1	28	50	Null	Null
	2	35	45	Low	Low
	3	23	50	Null	Null
10	1	22.8	55	Null	Null
	2	25.7	60	High	Low

B) Punctual weather conditions (temperature, hygrometry, cloud layer and wind strength) at the time of the bees release for each lab and run during the homing flight ring test 2017

Lab	Run	Mean temperature (°C)	Mean hygrometry (%)	Cloud layer	Wind strength
1A	**1**	29	35	Null	Low
	2	30	46	Low	Low
	3	30	24	Low	Null
1B	**1**	25	37	Low	Null
	2	30	40	Low	Null
	3	29	41	Low	Null
2	**1**	27.1	41	Low	Average
	2	33.6	33.2	Low	Average
	3	25.3	42.2	High	Average
3	**1**	26	57	High	Null
	2	26	47	Low	Null
	3	25.5	46	Average	Low
4	**1**	35.1	21	Null	Low
	2	36.2	27	Null	Low
	3	36.9	34	Null	Low
5	**1**	32	38	Null	Null
	2	33	46	Null	Null
	3	34	33	Low	Null
6	**1**	31	45	Null	Low
	2	30	38	Low	Low
	3	34	35	Null	Null
7	**1**	30.4	57.1	Low	Low
	2	35	33.6	Low	Low
	3	29.7	45.6	Low	Null
8	**1**	25.4	57	Low	Low

APPENDIX 9

Detail of the statistical analysis performed on homing success and homing duration 24 hours after the release of the bees

2016 – HOMING SUCCESS

Lab 1A

fisher.test (tab_cont, alternative='two.sided')
Fisher's Exact Test for Count Data
data: tab_cont
p-value = **0.02738**
alternative hypothesis: two.sided

***Multiple comparisons after Fisher Exact test (without P value adjustment)**

fisher.multcomp(tab_cont, p.method = "none")
Pairwise comparisons using Fisher's exact test for count data
data: tab_cont

	Cont	Thiam0.1	Thiam0.3
Thiam0.1	1.0000	-	-
Thiam0.3	0.2787	0.2786	-
Thiam1	0.1361	0.1369	**0.004376**

***P value adjustment method : Bonferroni**

> fisher.multcomp(tab_cont, p.method = "bonferroni")
Pairwise comparisons using Fisher's exact test for count data
data: tab_cont

	Cont	Thiam0.1	Thiam0.3
Thiam0.1	1.0000	-	-
Thiam0.3	1.0000	1.0000	-
Thiam1	0.8163	0.8211	**0.02625**

Lab 1B

fisher.test (tab_cont, alternative='two.sided')
Fisher's Exact Test for Count Data
data: tab_cont
p-value = **0.001563**
alternative hypothesis: two.sided

***Multiple comparisons after Fisher Exact test (without P value adjustment)**

fisher.multcomp(tab_cont, p.method = "none")
Pairwise comparisons using Fisher's exact test for count data
data: tab_cont

	Cont	Thiam0.1	Thiam0.3
Thiam0.1	1.000000	-	-
Thiam0.3	1.000000	1.000000	-
Thiam1	**0.002431**	**0.009732**	**0.009368**

***P value adjustment method : Bonferroni**

```
> fisher.multcomp(tab_cont, p.method = "bonferroni")
Pairwise comparisons using Fisher's exact test for count data
data:  tab_cont

          Cont    Thiam0.1 Thiam0.3
Thiam0.1  1.00000 -        -
Thiam0.3  1.00000 1.00000  -
Thiam1    0.01459 0.05839  0.05621
```

Lab 2

```
prop.test(tab_cont, alternative='two.sided',conf.level=.95,correct=FALSE)
4-sample test for equality of proportions without continuity correction
data:  tab_cont
X-squared = 23.9214, df = 3, p-value = 2.594e-05
alternative hypothesis: two.sided
sample estimates:
   prop 1    prop 2    prop 3    prop 4
0.3240741 0.3428571 0.3627451 0.6292135
```

***Multiple comparisons after Chi² test (without P value adjustment)**

```
pairwise.prop.test(tab_cont, p.adj = "none")
Pairwise comparisons using Pairwise comparison of proportions
data:  tab_cont
          Cont    Thiam0.1 Thiam0.3
Thiam0.1  0.88442 -        -
Thiam0.3  0.65659 0.87767  -
Thiam1    3.6e-05 0.00013  0.00042
```

***P value adjustment method: Bonferroni**

```
> pairwise.prop.test(tab_cont, p.adj = "bonferroni")
Pairwise comparisons using Pairwise comparison of proportions
data:  tab_cont

          Cont    Thiam0.1 Thiam0.3
Thiam0.1  1.00000 -        -
Thiam0.3  1.00000 1.00000  -
Thiam1    0.00022 0.00075  0.00249
```

Lab 3

prop.test(tab_cont, alternative='two.sided',conf.level=.95,correct=FALSE)

4-sample test for equality of proportions without continuity correction

data: tab_cont

X-squared = 20.0312, df = 3, p-value = 0.0001672

alternative hypothesis: two.sided

sample estimates:

prop 1	prop 2	prop 3	prop 4
0.07476636	0.13888889	0.20370370	0.29906542

***Multiple comparisons after Chi² test (without P value adjustment)**

pairwise.prop.test(tab_cont, p.adj = "none")

Pairwise comparisons using Pairwise comparison of proportions

data: tab_cont

	Cont	Thiam0.1	Thiam0.3
Thiam0.1	0.1935	-	-
Thiam0.3	0.0114	0.2786	-
Thiam1	**5.5e-05**	**0.0074**	**0.1457**

***P value adjustment method: Bonferroni**

> pairwise.prop.test(tab_cont, p.adj = "bonferroni")

Pairwise comparisons using Pairwise comparison of proportions

data: tab_cont

	Cont	Thiam0.1	Thiam0.3
Thiam0.1	1.00000	-	-
Thiam0.3	0.06820	1.00000	-
Thiam1	**0.00033**	**0.04466**	0.87431

Lab 4

prop.test(tab_cont, alternative='two.sided',conf.level=.95,correct=FALSE)

4-sample test for equality of proportions without continuity correction

data: tab_cont

X-squared = 1.2186, df = 3, p-value = 0.7485

alternative hypothesis: two.sided

sample estimates:

prop 1	prop 2	prop 3	prop 4
0.3409091	0.2674419	0.3176471	0.2941176

***Multiple comparisons after Chi² test (without P value adjustment)**

pairwise.prop.test(tab_cont, p.adj = "none")
Pairwise comparisons using Pairwise comparison of proportions
data: tab_cont

	Cont	Thiam0.1	Thiam0.3
Thiam0.1	0.37	-	-
Thiam0.3	0.87	0.58	-
Thiam1	0.62	0.83	0.87

Lab 5

prop.test(tab_cont, alternative='two.sided',conf.level=.95,correct=FALSE)
4-sample test for equality of proportions without continuity correction
data: tab_cont
X-squared = 7.9343, df = 3, p-value = 0.04739
alternative hypothesis: two.sided
sample estimates:

prop 1	prop 2	prop 3	prop 4
0.12396694	0.09756098	0.13821138	0.21848739

***Multiple comparisons after Chi² test (without P value adjustment)**

pairwise.prop.test(tab_cont, p.adj = "none")
Pairwise comparisons using Pairwise comparison of proportions
data: tab_cont

	Cont	Thiam0.1	Thiam0.3
Thiam0.1	0.650	-	-
Thiam0.3	0.889	0.429	-
Thiam1	**0.076**	**0.016**	**0.143**

***P value adjustment method: Bonferroni**

> pairwise.prop.test(tab_cont, p.adj = "bonferroni")
Pairwise comparisons using Pairwise comparison of proportions
data: tab_cont

	Cont	Thiam0.1	Thiam0.3
Thiam0.1	1.000	-	-
Thiam0.3	1.000	1.000	-
Thiam1	0.457	0.096	0.857

Lab 7

prop.test(tab_cont, alternative='two.sided',conf.level=.95,correct=FALSE)
4-sample test for equality of proportions without continuity correction
data: tab_cont
X-squared = 71.383, df = 3, p-value = 2.158e-15
alternative hypothesis: two.sided
sample estimates:

prop 1	prop 2	prop 3	prop 4
0.1500000	0.1807229	0.1625000	0.6666667

***Multiple comparisons after Chi² test (without p value adjustment)**

pairwise.prop.test(tab_cont, p.adj = "none")
Pairwise comparisons using Pairwise comparison of proportions
data: tab_cont

	Cont	Thiam0.1	Thiam0.3
Thiam0.1	0.75	-	-
Thiam0.3	1.00	0.92	-
Thiam1	**1.6e-10**	**1.6e-09**	**4.9e-10**

***P value adjustment method: Bonferroni**

> pairwise.prop.test(tab_cont, p.adj = "bonferroni")
Pairwise comparisons using Pairwise comparison of proportions
data: tab_cont

	Cont	Thiam0.1	Thiam0.3
Thiam0.1	1	-	-
Thiam0.3	1	1	-
Thiam1	**9.5e-10**	**9.4e-09**	**2.9e-09**

Lab 9

prop.test(tab_cont, alternative='two.sided',conf.level=.95,correct=FALSE)
4-sample test for equality of proportions without continuity correction
data: tab_cont
X-squared = 24.831, df = 3, p-value = 1.675e-05
alternative hypothesis: two.sided
sample estimates:

prop 1	prop 2	prop 3	prop 4
0.2631579	0.2637363	0.2708333	0.5494505

***Multiple comparisons after Chi² test (without p value adjustment)**

pairwise.prop.test(tab_cont, p.adj = "none")
Pairwise comparisons using Pairwise comparison of proportions
data: tab_cont

	Cont	Thiam0.1	Thiam0.3
Thiam0.1	1.00000	-	-
Thiam0.3	1.00000	1.00000	-
Thiam1	**0.00013**	**0.00016**	**0.00019**

***P value adjustment method: Bonferroni**

> pairwise.prop.test(tab_cont, p.adj = "bonferroni")
Pairwise comparisons using Pairwise comparison of proportions
data: tab_cont

	Cont	Thiam0.1	Thiam0.3
Thiam0.1	1.00000	-	-
Thiam0.3	1.00000	1.00000	-
Thiam1	**0.00077**	**0.00097**	**0.00116**

2017 – HOMING SUCCESS

Lab 1A

prop.test(tab_cont, alternative='two.sided',conf.level=.95,correct=FALSE)
4-sample test for equality of proportions without continuity correction
data: tab_cont
X-squared = 29.883, df = 3, p-value = 1.461e-06
alternative hypothesis: two.sided
sample estimates:

prop 1	prop 2	prop 3	prop 4
0.1444444	0.1444444	0.2222222	0.4444444

***Multiple comparisons after Chi² test (without p value adjustment)**

pairwise.prop.test(tab_cont, p.adj = "none")
Pairwise comparisons using Pairwise comparison of proportions
data: tab_cont

	Cont	Thiam0.1	Thiam0.3
Thiam0.1	1.0000	-	-
Thiam0.3	0.2478	0.2478	-
Thiam1	**2.1e-05**	**2.1e-05**	**0.0027**

***P value adjustment method : Bonferroni**

> pairwise.prop.test(tab_cont, p.adj = "bonferroni")
Pairwise comparisons using Pairwise comparison of proportions
data: tab_cont

	Cont	Thiam0.1	Thiam0.3
Thiam0.1	1.00000	-	-
Thiam0.3	1.00000	1.00000	-
Thiam1	**0.00013**	**0.00013**	**0.01598**

Lab 1B

prop.test(tab_cont, alternative='two.sided',conf.level=.95,correct=FALSE)
4-sample test for equality of proportions without continuity correction
data: tab_cont
X-squared = 6.4965, df = 3, p-value = 0.0898
alternative hypothesis: two.sided
sample estimates:

prop 1	prop 2	prop 3	prop 4
0.13333333	0.08888889	0.11111111	0.21111111

Lab 2

prop.test(tab_cont, alternative='two.sided',conf.level=.95,correct=FALSE)
4-sample test for equality of proportions without continuity correction
data: tab_cont
X-squared = 41.777, df = 3, p-value = 4.474e-09
alternative hypothesis: two.sided
sample estimates:

prop 1	prop 2	prop 3	prop 4
0.3611111	0.3675214	0.3243243	0.7037037

***Multiple comparisons after Chi² test (without p value adjustment)**

pairwise.prop.test(tab_cont, p.adj = "none")
Pairwise comparisons using Pairwise comparison of proportions
data: tab_cont

	Cont	Thiam0.1	Thiam0.3
Thiam0.1	1.00	-	-
Thiam0.3	0.67	0.59	-
Thiam1	**9.1e-07**	**8.9e-07**	**4.3e-08**

***P value adjustment method: Bonferroni**

> pairwise.prop.test(tab_cont, p.adj = "bonferroni")
Pairwise comparisons using Pairwise comparison of proportions
data: tab_cont

	Cont	Thiam0.1	Thiam0.3
Thiam0.1	1	-	-
Thiam0.3	1	1	-
Thiam1	**5.5e-06**	**5.4e-06**	**2.6e-07**

Lab 3

prop.test(tab_cont, alternative='two.sided',conf.level=.95,correct=FALSE)
4-sample test for equality of proportions without continuity correction
data: tab_cont
X-squared = 13.448, df = 3, p-value = 0.003762
alternative hypothesis: two.sided
sample estimates:

prop 1	prop 2	prop 3	prop 4
0.1111111	0.1226415	0.1495327	0.2752294

***Multiple comparisons after Chi² test (without p value adjustment)**

pairwise.prop.test(tab_cont, p.adj = "none")
Pairwise comparisons using Pairwise comparison of proportions
data: tab_cont

	Cont	Thiam0.1	Thiam0.3
Thiam0.1	0.9603	-	-
Thiam0.3	0.5259	0.7096	-
Thiam1	**0.0039**	**0.0086**	**0.0366**

***P value adjustment method: Bonferroni**

> pairwise.prop.test(tab_cont, p.adj = "bonferroni")
Pairwise comparisons using Pairwise comparison of proportions
data: tab_cont

	Cont	Thiam0.1	Thiam0.3
Thiam0.1	1.000	-	-
Thiam0.3	1.000	1.000	-
Thiam1	**0.023**	0.052	0.220

Lab 4

prop.test(tab_cont, alternative='two.sided',conf.level=.95,correct=FALSE)
4-sample test for equality of proportions without continuity correction
data: tab_cont
X-squared = 3.0341, df = 3, p-value = 0.3864
alternative hypothesis: two.sided
sample estimates:

prop 1	prop 2	prop 3	prop 4
0.1279070	0.1839080	0.1411765	0.2159091

***Multiple comparisons after Chi² test (without p value adjustment)**

pairwise.prop.test(tab_cont, p.adj = "none")
Pairwise comparisons using Pairwise comparison of proportions
data: tab_cont

	Cont	Thiam0.1	Thiam0.3
Thiam0.1	0.42	-	-
Thiam0.3	0.98	0.58	-
Thiam1	0.18	0.73	0.28

Lab 6

prop.test(tab_cont, alternative='two.sided',conf.level=.95,correct=FALSE)
4-sample test for equality of proportions without continuity correction
data: tab_cont
X-squared = 9.9708, df = 3, p-value = 0.01882
alternative hypothesis: two.sided
sample estimates:

prop 1	prop 2	prop 3	prop 4
0.2173913	0.1862745	0.1428571	0.3380282

***Multiple comparisons after Chi² test (without p value adjustment)**

pairwise.prop.test(tab_cont, p.adj = "none")
Pairwise comparisons using Pairwise comparison of proportions
data: tab_cont

	Cont	Thiam0.1	Thiam0.3
Thiam0.1	0.7184	-	-
Thiam0.3	0.2501	0.5245	-
Thiam1	0.1230	**0.0363**	**0.0049**

***P value adjustment method : Bonferroni**

> pairwise.prop.test(tab_cont, p.adj = "bonferroni")
Pairwise comparisons using Pairwise comparison of proportions
data: tab_cont

	Cont	Thiam0.1	Thiam0.3
Thiam0.1	1.000	-	-
Thiam0.3	1.000	1.000	-
Thiam1	0.738	0.218	**0.029**

Lab 7

prop.test(tab_cont, alternative='two.sided',conf.level=.95,correct=FALSE)
4-sample test for equality of proportions without continuity correction
data: tab_cont
X-squared = 104.19, df = 3, p-value < 2.2e-16
alternative hypothesis: two.sided
sample estimates:

prop 1	prop 2	prop 3	prop 4
0.1025641	0.1981982	0.1111111	0.6146789

***Multiple comparisons after Chi² test (without p value adjustment)**

pairwise.prop.test(tab_cont, p.adj = "none")
Pairwise comparisons using Pairwise comparison of proportions
data: tab_cont

	Cont	Thiam0.1	Thiam0.3
Thiam0.1	0.066	-	-
Thiam0.3	1.000	0.101	-
Thiam1	**2.2e-15**	**7.5e-10**	**7.8e-15**

***P value adjustment method: Bonferroni**

> pairwise.prop.test(tab_cont, p.adj = "bonferroni")
Pairwise comparisons using Pairwise comparison of proportions
data: tab_cont

	Cont	Thiam0.1	Thiam0.3
Thiam0.1	0.39	-	-
Thiam0.3	1.00	0.61	-
Thiam1	**1.3e-14**	**4.5e-09**	**4.7e-14**

2016 – HOMING DURATION

Lab 1A

kruskal.test(ab$rfid_release_min~ab$Treat)
Kruskal-Wallis rank sum test
data: ab$rfid_release_min by ab$Treat
Kruskal-Wallis chi-squared = 23.916, df = 3, p-value = 2.601e-05

***Multiple comparisons with Mann-Whitney test (without p value adjustment)**

pairwise.wilcox.test(ab$rfid_release_min,ab$Treat,p.adjust.method="none")
Pairwise comparisons using Wilcoxon rank sum test
data: ab$rfid_release_min and ab$Treat

	Cont	Thiam0.1	Thiam0.3
Thiam0.1	0.38527	-	-
Thiam0.3	0.30891	0.85934	-
Thiam1	**8.6e-06**	**0.00018**	**0.00021**

***P value adjustment method : Bonferroni**

>pairwise.wilcox.test(ab$rfid_release_min,ab$Treat,p.adjust.method="bonferroni")
Pairwise comparisons using Wilcoxon rank sum test
data: ab$rfid_release_min and ab$Treat

	Cont	Thiam0.1	Thiam0.3
Thiam0.1	1.0000	-	-
Thiam0.3	1.0000	1.0000	-
Thiam1	**5.2e-05**	**0.0011**	**0.0012**

Lab 1B

kruskal.test(ab$rfid_release_min~ab$Treat)
Kruskal-Wallis rank sum test
data: ab$rfid_release_min by ab$Treat
Kruskal-Wallis chi-squared = 22.1207, df = 3, p-value = 6.157e-05

***Multiple comparisons with Mann-Whitney test (without p value adjustment)**

pairwise.wilcox.test(ab$rfid_release_min,ab$Treat,p.adjust.method="none")
Pairwise comparisons using Wilcoxon rank sum test
data: ab$rfid_release_min and ab$Treat

	Cont	Thiam0.1	Thiam0.3
Thiam0.1	0.31786	-	-
Thiam0.3	0.07116	0.51686	-
Thiam1	**6.7e-06**	**0.00092**	**0.00230**

***P value adjustment method : Bonferroni**

> pairwise.wilcox.test(ab$rfid_release_min,ab$Treat,p.adjust.method="bonferroni")
Pairwise comparisons using Wilcoxon rank sum test
data: ab$rfid_release_min and ab$Treat

	Cont	Thiam0.1	Thiam0.3
Thiam0.1	1.0000	-	-
Thiam0.3	0.4270	1.0000	-
Thiam1	**4e-05**	**0.0055**	**0.0138**

Lab 2

kruskal.test(ab$rfid_release_min~ab$Treat)
Kruskal-Wallis rank sum test
data: ab$rfid_release_min by ab$Treat
Kruskal-Wallis chi-squared = 10.9258, df = 3, p-value = 0.01213

***Multiple comparisons with Mann-Whitney test (without p value adjustment)**

pairwise.wilcox.test(ab$rfid_release_min,ab$Treat,p.adjust.method="none")
Pairwise comparisons using Wilcoxon rank sum test
data: ab$rfid_release_min and ab$Treat

	Cont	Thiam0.1	Thiam0.3
Thiam0.1	0.4502	-	-
Thiam0.3	0.8796	0.4001	-
Thiam1	0.0022	0.0176	0.0032

***P value adjustment method : Bonferroni**

> pairwise.wilcox.test(ab$rfid_release_min,ab$Treat,p.adjust.method="bonferroni")
Pairwise comparisons using Wilcoxon rank sum test
data: ab$rfid_release_min and ab$Treat

	Cont	Thiam0.1	Thiam0.3
Thiam0.1	1.000	-	-
Thiam0.3	1.000	1.000	-
Thiam1	**0.013**	0.105	**0.019**

Lab 3

kruskal.test(ab$rfid_release_min~ab$Treat)
Kruskal-Wallis rank sum test
data: ab$rfid_release_min by ab$Treat
Kruskal-Wallis chi-squared = 3.3861, df = 3, p-value = 0.3358

Lab 4

kruskal.test(ab$rfid_release_min~ab$Treat)
Kruskal-Wallis rank sum test
data: ab$rfid_release_min by ab$Treat
Kruskal-Wallis chi-squared = 1.4074, df = 3, p-value = 0.7038

Lab 5

kruskal.test(ab$rfid_release_min~ab$Treat)
Kruskal-Wallis rank sum test
data: ab$rfid_release_min by ab$Treat
Kruskal-Wallis chi-squared = 6.7554, df = 3, p-value = 0.08012

Lab 7

kruskal.test(ab$rfid_release_min~ab$Treat)
Kruskal-Wallis rank sum test
data: ab$rfid_release_min by ab$Treat
Kruskal-Wallis chi-squared = 3.8146, df = 3, p-value = 0.2822

Lab 9

kruskal.test(ab$rfid_release_min~ab$Treat)
Kruskal-Wallis rank sum test
data: ab$rfid_release_min by ab$Treat
Kruskal-Wallis chi-squared = 8.7884, df = 3, p-value = 0.03224

***Multiple comparisons with Mann-Whitney test (without p value adjustment)**

pairwise.wilcox.test(ab$rfid_release_min,ab$Treat,p.adjust.method="none")
Pairwise comparisons using Wilcoxon rank sum test
data: ab$rfid_release_min and ab$Treat

	Cont	Thiam0.1	Thiam0.3
Thiam0.1	0.2798	-	-
Thiam0.3	0.5210	0.6388	-
Thiam1	0.0039	0.0250	0.0404

***P value adjustment method: Bonferroni**

> pairwise.wilcox.test(ab$rfid_release_min,ab$Treat,p.adjust.method="bonferroni")
Pairwise comparisons using Wilcoxon rank sum test
data: ab$rfid_release_min and ab$Treat

	Cont	Thiam0.1	Thiam0.3
Thiam0.1	1.000	-	-
Thiam0.3	1.000	1.000	-
Thiam1	**0.024**	0.150	0.242

2017 – HOMING DURATION

Lab 1A

kruskal.test(ab$rfid_release_min~ab$Treat)
Kruskal-Wallis rank sum test
data: ab$rfid_release_min by ab$Treat
Kruskal-Wallis chi-squared = 10.374, df = 3, p-value = 0.01564

***Multiple comparisons with Mann-Whitney test (without p value adjustment)**

pairwise.wilcox.test(ab$rfid_release_min,ab$Treat,p.adjust.method="none")
Pairwise comparisons using Wilcoxon rank sum test
data: ab$rfid_release_min and ab$Treat

	Cont	Thiam0.1	Thiam0.3
Thiam0.1	0.6541	-	-
Thiam0.3	0.7198	0.8920	-
Thiam1	0.0013	0.0143	0.0157

***P value adjustment method: Bonferroni**

> pairwise.wilcox.test(ab$rfid_release_min,ab$Treat,p.adjust.method="bonferroni")
Pairwise comparisons using Wilcoxon rank sum test
data: ab$rfid_release_min and ab$Treat

	Cont	Thiam0.1	Thiam0.3
Thiam0.1	1.0000	-	-
Thiam0.3	1.0000	1.0000	-
Thiam1	**0.0076**	0.0857	0.0940

Lab 1B

kruskal.test(ab$rfid_release_min~ab$Treat)
Kruskal-Wallis rank sum test
data: ab$rfid_release_min by ab$Treat
Kruskal-Wallis chi-squared = 8.1092, df = 3, p-value = 0.04381

***Multiple comparisons with Mann-Whitney test (without p value adjustment)**

pairwise.wilcox.test(ab$rfid_release_min,ab$Treat,p.adjust.method="none")
Pairwise comparisons using Wilcoxon rank sum test
data: ab$rfid_release_min and ab$Treat

	Cont	Thiam0.1	Thiam0.3
Thiam0.1	0.045	-	-
Thiam0.3	0.493	0.171	-
Thiam1	0.022	0.316	0.050

***P value adjustment method: Bonferroni**

> pairwise.wilcox.test(ab$rfid_release_min,ab$Treat,p.adjust.method="bonferroni")
Pairwise comparisons using Wilcoxon rank sum test
data: ab$rfid_release_min and ab$Treat

	Cont	Thiam0.1	Thiam0.3
Thiam0.1	0.27	-	-
Thiam0.3	1.00	1.00	-
Thiam1	0.13	1.00	0.30

Lab 2

kruskal.test(ab$rfid_release_min~ab$Treat)
Kruskal-Wallis rank sum test
data: ab$rfid_release_min by ab$Treat
Kruskal-Wallis chi-squared = 5.6527, df = 3, p-value = 0.1298

Lab 3

kruskal.test(ab$rfid_release_min~ab$Treat)
Kruskal-Wallis rank sum test
data: ab$rfid_release_min by ab$Treat
Kruskal-Wallis chi-squared = 6.8323, df = 3, p-value = 0.07744

Lab 4

kruskal.test(ab$rfid_release_min~ab$Treat)
Kruskal-Wallis rank sum test
data: ab$rfid_release_min by ab$Treat
Kruskal-Wallis chi-squared = 6.8593, df = 3, p-value = 0.07652

Lab 6

kruskal.test(ab$rfid_release_min~ab$Treat)
Kruskal-Wallis rank sum test
data: ab$rfid_release_min by ab$Treat
Kruskal-Wallis chi-squared = 12.794, df = 3, p-value = 0.005104

***Multiple comparisons with Mann-Whitney test (without p value adjustment)**

pairwise.wilcox.test(ab$rfid_release_min,ab$Treat,p.adjust.method="none")
Pairwise comparisons using Wilcoxon rank sum test
data: ab$rfid_release_min and ab$Treat

	Cont	Thiam0.1	Thiam0.3
Thiam0.1	0.15748	-	-
Thiam0.3	0.72488	0.33855	-
Thiam1	**0.00078**	**0.01560**	**0.00440**

***P value adjustment method: Bonferroni**

> pairwise.wilcox.test(ab$rfid_release_min,ab$Treat,p.adjust.method="bonferroni")
Pairwise comparisons using Wilcoxon rank sum test
data: ab$rfid_release_min and ab$Treat

	Cont	Thiam0.1	Thiam0.3
Thiam0.1	0.9449	-	-
Thiam0.3	1.0000	1.0000	-
Thiam1	**0.0047**	0.0936	**0.0264**

Lab 7

kruskal.test(ab$rfid_release_min~ab$Treat)
Kruskal-Wallis rank sum test
data: ab$rfid_release_min by ab$Treat
Kruskal-Wallis chi-squared = 25.267, df = 3, p-value = 1.358e-05

***Multiple comparisons with Mann-Whitney test (without p value adjustment)**

pairwise.wilcox.test(ab$rfid_release_min,ab$Treat,p.adjust.method="none")
Pairwise comparisons using Wilcoxon rank sum test
data: ab$rfid_release_min and ab$Treat

	Cont	Thiam0.1	Thiam0.3
Thiam0.1	0.16466	-	-
Thiam0.3	0.64730	0.03949	-
Thiam1	1.3e-05	0.00086	4.3e-06

***P value adjustment method: Bonferroni**

> pairwise.wilcox.test(ab$rfid_release_min,ab$Treat,p.adjust.method="bonferroni")
Pairwise comparisons using Wilcoxon rank sum test
data: ab$rfid_release_min and ab$Treat

	Cont	Thiam0.1	Thiam0.3
Thiam0.1	0.9879	-	-
Thiam0.3	1.0000	0.2369	-
Thiam1	**8.1e-05**	**0.0052**	**2.6e-05**

APPENDIX 10

Detail of the generalized linear mixed effect models (GLMMs) performed to assess the effect of thiamethoxam dose, mean temperature 24h-after release (Temp) as well as their interactions on honeybee homing performances (experiments 2016 and 2017)

2016

A) All test runs considered

succes2<- glmer(Retour~Doser*Tempr+(1|Sitef)+(1|Sitef:Hive), family=binomial, data=res1, na.action="na.fail")
summary(succes2)

Generalized linear mixed model fit by maximum likelihood (Laplace Approximation) [glmerMod]
Family: binomial (logit)
Formula: Retour ~ Doser * Tempr + (1 | Sitef) + (1 | Sitef:Hive)
Data: res1

```
     AIC      BIC   logLik deviance df.resid
  3459.3   3496.6  -1723.7   3447.3     3672
```

Scaled residuals:

```
    Min      1Q  Median      3Q     Max
-7.4769 -0.4127  0.2926  0.5428  3.2688
```

Random effects:

```
 Groups     Name        Variance Std.Dev.
 Sitef:Hive (Intercept) 0.4274   0.6537
 Sitef      (Intercept) 1.5380   1.2402
```

Number of obs: 3678, groups: Sitef:Hive, 29; Sitef, 10

Fixed effects:

```
            Estimate Std. Error z value Pr(>|z|)
(Intercept)   1.7711     0.5534   3.200  0.00137 **
Doser        -3.0626     0.2812 -10.892  < 2e-16 ***
Tempr        -0.4274     0.9222  -0.463  0.64305
```

Doser:Tempr 3.4670 0.6279 5.522 3.36e-08 *

Signif. codes: 0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1

Correlation of Fixed Effects:

```
            (Intr) Doser  Tempr
Doser       -0.176
Tempr       -0.658  0.191
Doser:Tempr  0.140 -0.830 -0.209
```

B) Valid test runs considered

succes2<- glmer(Retour~Doser*Tempr+(1|Sitef)+(1|Sitef:Hive), family=binomial, data=res1, na.action="na.fail")
summary(succes2)

Generalized linear mixed model fit by maximum likelihood (Laplace Approximation) [glmerMod]
Family: binomial (logit)
Formula: Retour ~ Doser * Tempr + (1 | Sitef) + (1 | Sitef:Hive)
Data: res1

AIC	BIC	logLik	deviance	df.resid
2814.2	2850.5	-1401.1	2802.2	3116

Scaled residuals:

Min	1Q	Median	3Q	Max
-7.3041	0.1803	0.3363	0.5301	1.4405

Random effects:

Groups	Name	Variance	Std.Dev.
Sitef:Hive	(Intercept)	0.3239	0.5692
Sitef	(Intercept)	0.7611	0.8724

Number of obs: 3122, groups: Sitef:Hive, 24; Sitef, 9

Fixed effects:

	Estimate	Std. Error	z value	Pr(>|z|)	
(Intercept)	2.3431	0.4656	5.032	4.85e-07	***
Doser	-3.0534	0.2998	-10.185	< 2e-16	***
Tempr	-0.7408	0.8195	-0.904	0.366	
Doser:Tempr	**3.5196**	**0.6286**	**5.599**	**2.15e-08**	***

Signif. codes: 0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1

Correlation of Fixed Effects:

	(Intr)	Doser	Tempr
Doser	-0.244		
Tempr	-0.715	0.232	
Doser:Tempr	0.191	-0.830	-0.249

2017

A) All test runs considered

succes2<- glmer(Retour~Doser*Tempr+(1|Sitef)+(1|Sitef:Hive), family=binomial, data=res1, na.action="na.fail")
summary(succes2)

Generalized linear mixed model fit by maximum likelihood (Laplace Approximation) [glmerMod]
Family: binomial (logit)
Formula: Retour ~ Doser * Tempr + (1 | Sitef) + (1 | Sitef:Hive)
Data: res1

AIC	BIC	logLik	deviance	df.resid
2761.4	2796.6	-1374.7	2749.4	2597

Scaled residuals:

Min	1Q	Median	3Q	Max
-3.9493	-0.8236	0.4295	0.6016	1.4459

Random effects:

Groups	Name	Variance	Std.Dev.
Sitef:Hive	(Intercept)	0.1517	0.3895
Sitef	(Intercept)	0.2601	0.5100

Number of obs: 2603, groups: Sitef:Hive, 20; Sitef, 7

Fixed effects:

	Estimate	Std. Error	z value	Pr(>|z|)	
(Intercept)	1.9291	0.3539	5.450	5.03e-08	***
Doser	-4.0281	0.5352	-7.527	5.21e-14	***
Tempr	-0.5450	0.5419	-1.006	0.3146	
Doser:Tempr	2.0532	0.9239	2.222	0.0263	*

Signif. codes: 0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1

Correlation of Fixed Effects:

	(Intr)	Doser	Tempr
Doser	-0.326		
Tempr	-0.775	0.321	
Doser:Tempr	0.292	-0.888	-0.364

B) Valid test runs considered

```
succes2<- glmer(Retour~Doser*Tempr+(1|Sitef)+(1|Sitef:Hive), family=binomial, data=res1, na.action="na.fail")
summary(succes2)

Generalized linear mixed model fit by maximum likelihood (Laplace Approximation) [glmerMod]
Family: binomial  ( logit )
Formula: Retour ~ Doser * Tempr + (1 | Sitef) + (1 | Sitef:Hive)
Data: res1

     AIC      BIC   logLik deviance df.resid
  2467.9   2502.6  -1228.0   2455.9     2395

Scaled residuals:
    Min      1Q  Median      3Q     Max
-3.9263 -0.6793  0.4158  0.5707  1.4720

Random effects:
 Groups     Name        Variance Std.Dev.
 Sitef:Hive (Intercept) 0.1651   0.4063
 Sitef      (Intercept) 0.2606   0.5105
Number of obs: 2401, groups:  Sitef:Hive, 18; Sitef, 7

Fixed effects:
            Estimate Std. Error z value Pr(>|z|)
(Intercept)   1.9508     0.3622   5.386 7.22e-08 ***
Doser        -3.9263     0.5385  -7.291 3.08e-13 ***
Tempr        -0.3081     0.5626  -0.548    0.584
Doser:Tempr   1.0272     0.9688   1.060    0.289
---
Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1

Correlation of Fixed Effects:
            (Intr) Doser  Tempr
Doser       -0.322
Tempr       -0.768  0.320
Doser:Tempr  0.282 -0.867 -0.372
```

Detail of the generalized linear mixed effect models (GLMMs) performed to assess the effect of thiamethoxam dose, varroa infestation (Varroa) as well as their interactions on honeybee homing performances (experiments 2017)

Valid test runs considered

```
succes2<- glmer(Retour~Doser*VarroaLg10r+(1|Sitef)+(1|Sitef:Hive), family=binomial, data=res1,
na.action="na.fail")
summary(succes2)


Generalized linear mixed model fit by maximum likelihood (Laplace
  Approximation) [glmerMod]
 Family: binomial  ( logit )
Formula: Retour ~ Doser * VarroaLg10r + (1 | Sitef) + (1 | Sitef:Hive)
   Data: res1

     AIC      BIC   logLik deviance df.resid
  1918.1   1951.2   -953.0   1906.1     1831

Scaled residuals:
    Min      1Q  Median      3Q     Max
-3.2628 -0.7103  0.4129  0.5736  1.4079

Random effects:
 Groups     Name        Variance Std.Dev.
 Sitef:Hive (Intercept) 0.003603 0.06003
 Sitef      (Intercept) 0.389696 0.62426
Number of obs: 1837, groups:  Sitef:Hive, 14; Sitef, 5

Fixed effects:
                 Estimate Std. Error z value Pr(>|z|)
(Intercept)       1.49534    0.29964   4.990 6.03e-07 ***
Doser            -2.70686    0.37371  -7.243 4.38e-13 ***
VarroaLg10r       0.54691    0.24736   2.211    0.027 *
Doser:VarroaLg10r -0.04956    0.87371  -0.057    0.955
---
Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1

Correlation of Fixed Effects:
            (Intr) Doser  VrrL10
Doser       -0.244
VarroaLg10r -0.216  0.333
Dsr:VrrLg10  0.129 -0.534 -0.626
```

APPENDIX 11

Detail of the statistical analysis performed on homing performances 24 hours after the release of the bees
Experiments 2017: Feeding *vs* no feeding before release

ITSAP - Test with candy *ad libitum*

prop.test(tab_cont, alternative='two.sided',conf.level=.95,correct=FALSE)
4-sample test for equality of proportions without continuitycorrection
data: tab_cont
X-squared = 28.831, df = 3, p-value = 2.431e-06
alternative hypothesis: two.sided
sample estimates:
prop 1 prop 2 prop 3 prop 4
0.2380952 0.2435897 0.5802469 0.2926829

***Multiple comparisons after Chi² test (without P value adjustment)**
pairwise.prop.test(tab_cont, p.adj = "none")
Pairwise comparisons using Pairwise comparison of proportions
data: tab_cont

	Cont	ContF	Thiam1
ContF	1.00000	-	-
Thiam1	1.6e-05	3.4e-05	-
Thiam1F	0.53472	0.60182	0.00039

F= fed ad libitum before release

***P value adjustment method: Bonferroni**
> pairwise.prop.test(tab_cont, p.adj = "bonferroni")
Pairwise comparisons using Pairwise comparison of proportions
data: tab_cont

	Cont	ContN	Thiam1
ContN	1.0000	-	-
Thiam1	**9.6e-05**	**0.0002**	-
Thiam1N	1.0000	1.0000	**0.0024**

ITSAP - Test with sucrose solution 30 % (w/v) *ad libitum*

prop.test(tab_cont, alternative='two.sided',conf.level=.95,correct=FALSE)
4-sample test for equality of proportions without continuity correction
data: tab_cont
X-squared = 11.031, df = 3, p-value = 0.01156
alternative hypothesis: two.sided
sample estimates:
prop 1 prop 2 prop 3 prop 4
0.1477273 0.2613636 0.3678161 0.2727273

***Multiple comparisons after Chi² test (without P value adjustment)**
pairwise.prop.test(tab_cont, p.adj = "none")
Pairwise comparisons using Pairwise comparison of proportions
data: tab_cont

	Cont	ContF	Thiam1
ContF	0.0926	-	-
Thiam1	**0.0016**	0.1758	-
Thiam1F	0.0643	1.0000	0.2355

F= fed ad libitum before release

***P value adjustment method : Bonferroni**
> pairwise.prop.test(tab_cont, p.adj = "bonferroni")
Pairwise comparisons using Pairwise comparison of proportions
data: tab_cont

	Cont	ContN	Thiam1
ContN	0.5556	-	-
Thiam1	**0.0095**	1.0000	-
Thiam1N	0.3860	1.0000	1.0000

INRA Le Magneraud - Test with candy *ad libitum*

prop.test(tab_cont, alternative='two.sided',conf.level=.95,correct=FALSE)
4-sample test for equality of proportions without continuity correction
data: tab_cont
X-squared = 57.57, df = 3, p-value = 1.941e-12
alternative hypothesis: two.sided
sample estimates:
prop 1 prop 2 prop 3 prop 4
0.7209302 0.3209877 0.8311688 0.7710843

***Multiple comparisons after Chi² test (without P value adjustment)**
pairwise.prop.test(tab_cont, p.adj = "none")
Pairwise comparisons using Pairwise comparison of proportions
data: tab_cont

	Cont	ContF	Thiam1
ContF	**5.2e-07**	-	-
Thiam1	0.14	**2.7e-10**	-
Thiam1F	0.57	**1.8e-08**	0.45

F= fed ad libitum before release

***P value adjustment method: Bonferroni**
> pairwise.prop.test(tab_cont, p.adj = "bonferroni")
Pairwise comparisons using Pairwise comparison of proportions
data: tab_cont

	Cont	ContN	Thiam1
ContN	**3.1e-06**	-	-
Thiam1	0.82	**1.6e-09**	-
Thiam1N	1.00	**1.1e-07**	1.00

APPENDIX 12

Detail of the statistical analyses performed to compare the crop content of foragers at each experimental site (ITSAP and INRA Le Magneraud)

Research and development study performed in 2017

ITSAP (Avignon, France)

kruskal.test(Jab1$Crop ~ Jab1$Treat)
Kruskal-Wallis rank sum test
data: Jab1$Crop by factor(Jab1$Treat)
Kruskal-Wallis chi-squared = 7.7866, df = 3, p-value = 0.05063

INRA Le Magneraud (Surgères, France)

kruskal.test(Jab1$Crop ~ Jab1$Treat)
Kruskal-Wallis rank sum test
data: Jab1$Crop by factor(Jab1$Treat)
Kruskal-Wallis chi-squared = 73.313, df = 3, p-value = 8.332e-16

***Mann-Whitney comparisons (without P value adjustment)**

pairwise.wilcox.test(Jab1$Crop, Jab1$Treat,p.adjust.method="none")
Pairwise comparisons using Wilcoxon rank sum test
data: Jab1$Crop and factor(Jab1$Treat)

	Cont	ContF	Thiam1
ContF	**3.0e-07**	-	-
Thiam1	0.60	**3.6e-09**	-
Thiam1F	**1.1e-09**	0.11	**1.5e-11**

F= fed with 10 µl per bee of sucrose solution 30% (w/v) before release

***P value adjustment with Bonferroni method**

> pairwise.prop.test(tab_cont, p.adj = "bonferroni")
Pairwise comparisons using Pairwise comparison of proportions
data: tab_cont

	Cont	ContF	Thiam1
ContF	**1.8e-06**	-	-
Thiam1	1.00	**2.2e-08**	-
Thiam1F	**6.6e-09**	0.66	**9e-11**

Part III - Final report of the international ring test 2018 and 2019 and overall conclusions from the several validation studies of the Homing flight test in honeybee (Apis mellifera L.) after single exposure to sublethal doses of a test chemical

Results of the international ring test 2018 and 2019
Final report and overall conclusions from the several validation studies

Validation of the Homing flight test in honeybee (Apis mellifera L.) after single exposure to sublethal doses of a test chemical

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31 March 2020

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1-INTRODUCTION

According to the Regulation (EC) No 1107/2009 (Annex II point 3.8.3), an active substance or a formulated plant protection product, shall only be approved if it is carefully evaluated following an appropriate risk assessment. Among several factors, this includes acute and chronic effects on honeybee colony survival and development, considering effects on honeybee larvae and **honeybee behaviour**. For the latter, no standardized method exists to evaluate sublethal effects on foraging behaviour of honeybees. Sublethal effects in individual worker bees may have the potential to affect functions at colony level and/or colony survival (Henry et al. 2012, 2015, Woodcock et al. 2017). Recent revision of plant protection products' risk assessment on bees recommended the use of a homing flight test to study the effect of sublethal doses of plant protection products on this trait of interest (EFSA, 2013).

The homing test proposes to assess effects of a single, oral exposure to sublethal doses of a chemical (technical grade active substance or a formulation) on the homing performance of forager bees. Thereby, feeding solutions are administered under controlled conditions and subsequently foragers are released in order to mimic field realistic homing conditions.
The method project was adopted by the OECD Working Group of National Coordinators of the Test Guidelines Programme (WNT) and is integrated in the work plan of OECD since 2016.

Background of the OECD ring test foundation is presented in the previous report ("Results of the international ring test 2016 and 2017") for the validation of a homing flight test design.
The test' endpoint is the determination of a No-Observed-Effect-Dose (NOED) on the homing success of foragers released at a distance of 1 km (+/-100 m) away from the experimental colony. This distance is within the range that foragers routinely cover during normal foraging flights (Steffan-Dewenter & Kuhn, 2003, Park & Nieh, 2017). Moreover, the proposed ring test aims to establish a validity criterion of the studies regarding the minimum- and acceptable homing-success-rate of untreated control bees.
The active substance thiamethoxam was used as a reference item in this ring test, since several studies have demonstrated that thiamethoxam can negatively affect the homing ability of foragers (Henry et al. 2012, 2014, 2015). For each trial (from 2015-2017) we tested three sublethal doses of the active substance (according to a geometric progression with a ratio of 3): 0.11 ng, 0.33 ng and 1 ng per bee. Tested doses range was changed in 2018 and 2019 to 0.33 ng, 1 ng and 1.5 ng per bee to take into account possible differences in sensitivity of the bees around the dose of 1 ng per bee. Similarly, a control solution (acetone 0.1 % in a 30 % w/v sucrose solution) was included. All labs used technical grade thiamethoxam originating from the same batch number (purity = 99%). For each test run, bees were exposed collectively (in 10 bees-cages) to one of the four feeding solutions.
Homing performance was measured (for 24 hours) by monitoring free-ranging foragers with radio-frequency identification (RFID) tagging technology. For each treatment-group, both, homing success rate and its corresponding duration were calculated from the automatically saved data. For the interpretation of obtained results, the variability and potential causing factors were discussed.

Methodological improvements were continuously achieved based on experimental observations. From 2016, one main improvement of the method consisted to use an alternative method to that of the Phacelia approach as described in the first report in 2015 ("Summary of results of the First international ring test 2015"). The alternative method is based on the use of a colored dye powder used to stain forager bees, which allows the identification of bees at the hive entrance which were released at a distance of 1 km (+/-100 m) from the test colony. Then, it can be ensured that foragers have at least one successful homing trip and thus advanced knowledge of the pathway back to the colony from the release site. This alternative greatly improved the test feasibility and was validated. The other main proposal was the addition of a feeding phase *ad libitum* before the tagged bees' release to facilitate the energy level of the bees. But this food supply could appear as a source of variability

of homing results in exposed bees due to the dilution of the remaining volume of sucrose from the exposure phase in the bee crop. As a result, this feeding phase was suppressed from 2018, but the protocol was adjusted for good maintenance and performance of the bees during the laboratory phase with **i**) a pre-exposure starvation duration fixed to 1h30; **ii**) an exposure starvation performed in dark conditions during 1h prolonged for a maximum of 30 min if needed; **iii**) a decrease of the post-exposure starvation period from 1 h to 40 min as for Henry et al. (2012).

Additionally, studies carried out in 2018 by two labs of the ring test, pointed out the need to focus as far as possible on pollen foragers instead of only nectar foragers. Indeed, pollen collectors are expected to have relatively low stomach content. This helps for a better consumption and homogeneous distribution of the sucrose solution by trophallaxis among the bees during the exposure phase, and this may prevent possible dilution that could occur when only nectar foragers with expected higher stomach content are collected. Consequently, increase in accuracy of the effects measured with the doses tested is expected. Focus on pollen collectors was adopted in 2019.

Then, the ring test trials were pursued and the corresponding results obtained during 2018 and 2019 are presented in this report. Overall considerations of the results over the five years of ring test are also included.

2-INFORMATION ON THE RING TEST GROUP

In total, ten laboratories participated in the ring tests of 2018 and 2019. Participants represented a wide range of stakeholder groups, including governmental institutions, contract laboratories and technical bee institutes.

Laboratory	**Responsible person(s)**
ITSAP-Institut de l'Abeille, **France** ***Project leader, organiser of the ring test***	Julie Fourrier
INRA Le Magneraud, **France** ***Co-organiser of the ring test***	Pierrick Aupinel Colombe Chevallereau Carole Moreau-Vauzelle
Innovative Environmental Services (IES) Ltd, **Switzerland**	Bettina Hodapp Stefan Kimmel
CREA-AA, **Italy**	Piotr Medrzycki Irene Guerra
Agroscope, Swiss Bee Research Centre **Switzerland**	Lukas Jeker Daniela Grossar Michael Eyer
Ibacon, Institut für Biologische Analytik und Consulting Gmbh, **Germany**	Martin Benz Stephan Schmitzer Tatsuya Sekine
The Fera (Science) Ltd, **United Kingdom**	Selwyn Wilkins Emma Wright
BioChem agrar GmbH, **Germany**	Markus Barth Melanie Hänsel Kristin Schmidt
LAVES Institute for Apidology Celle, **Germany**	Martina Janke Dorothee Lueken
TESTAPI, **France**	Hervé Giffard Olivier Mamet

Organisation and coordination of this work was supported by grants from the French Ministry of Agriculture (FranceAgriMer) and Lune de Miel® Foundation.

3-RING TEST SCHEDULE (2018 and 2019)

	2018	2019
Start of experimental phase	May	May
End of experimental phase	September	September
Evaluation of results	July to December	July to December
Results presentation to the ring test group	January 2019	January 2020

4-MATERIAL AND METHODS

4.1 Honeybees

Source of the colonies, treatments and health status: Chemical treatments (anti-varroa…) have been completed at least four weeks before the start of the experiment. Queen-right (queens with known history and not older than 2 years) and healthy colonies (as far as possible disease-free) were used for the experiments.

Hives characteristics: Each test hive was equipped with 10 to 12-frames. It has to be checked that bees correctly circulate through RFID readers to get in and out of the colony (no cluster of bees at the hive entrance) and that no trophallaxis between inside and outside bees occurs at the bottom of the hive. According to climatic conditions, hive volume can be increased by adding one to two supers and good thermoregulation during summer climatic conditions will be ensured.

In 2019, it was added that strong and active colonies with enough brood and food stock are used for the test.

Preparation of the colonies: The colonies used for the ring test were homogenous in terms of colony strength, food storage, amount of brood and experimental preparation. Hives with ten frames configuration comprised five to seven brood combs of all stages (eggs, uncapped larvae and pupae) and hives with twelve frames configuration contained six to eight brood combs. Each hive configurations contained two to three food combs and at least one empty frame. A colony inspection (routine apiarist visit) was performed for each experimental colony one to four days before the test start to prepare the colony and to verify health status. Good colony activity was checked by monitoring the foraging activity at the hive entrance.

Varroa load: During each apiarist visit, a sample of bees on brood frames (± 200-400 bees) was collected. All samples were sent to ITSAP Lab. Honeybees were washed with water and detergent (Dietemann et al. 2013) in order to count the phoretic mites (*Varroa destructor*) and establish the number of varroas per 100 honeybees (Lee et al. 2010). This counting is an indicator of the colony's Varroa load.

Installation of the colonies: the colonies used for the test had to be installed on the experimental site, at least one week before the start of the test, to allow acclimatisation and familiarisation with the environment by the honeybees. If all the colonies were placed on the experimental site at the same

time, they were separated spatially by few meters (± 10 meters) and placed in a staggered configuration to maximally avoid drift of labelled bees between the colonies.

4.2 RFID device

RFID (Radio Frequency Identification) device: The RFID technology (Streit *et al*. 2003; Decourtye *et al*. 2011) allows detection each time an RFID tagged bee passed through the reader (working distance of 3 mm). The principle depends on the emission of a radio signal from the reader which is received by the tag on the bee's thorax. The tag is not equipped with a power source (passive function) and it obtains its operating power from the reading process to emit a unique identification code. The reader automatically recognizes a virtually unlimited number of individual insects.

For the five ring test years, the used tags worked with 13.56 MHz frequency; Microsensys GmbH, Erfurt, Germany (2.0 x 1.7 x 0.5 mm).They weighed no more than 3 mg, equivalent to approximately 3 % of the weight of a worker bee. RFID tags were glued with dental cement (Temposil 2) on the dorsal side of the thorax of the bees. The used RFID system was MAJA system (Microsensys GmbH, Erfurt, Germany). It comprised of one Host (small computer with a Windows system) that recorded data of all forager passing's on a SD card. Four readers were placed at the entrance of the hive (parallel arrangement). Each reader spanned a tunnel of 14 × 21.5 mm (7 mm high) acting as an entrance to the colony. Readers were installed at the hive entrance thanks to an interface (in plastic or wood) between hive and readers (= mask). Then, the bees were able to enter the hive by passing through the 4 possible entrances formed by the readers (**Figure 1**, **Appendix 1**).

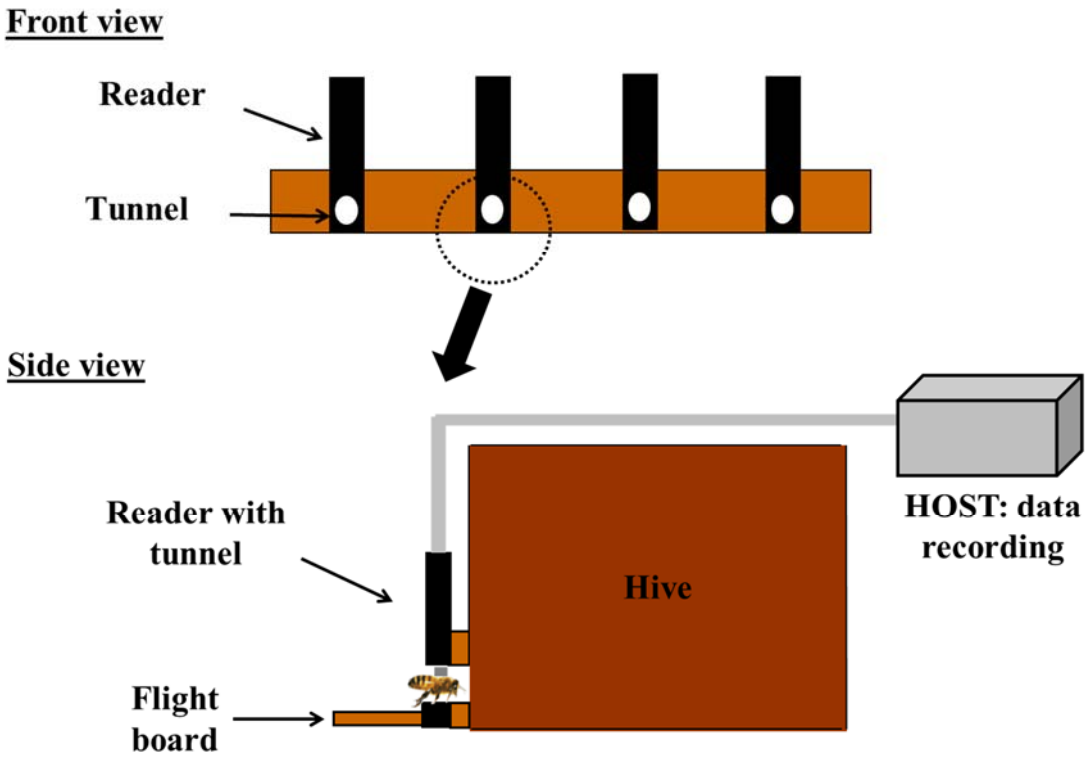


Figure 1: Picture of the RFID device

The tag's identification code (Unique identifications, or UIDs) and the exact time of the event (date, hour, minute and second) were recorded with the MAJA Host capture software. RFID data were collected by connecting the MAJA Host with a PC laptop equipped with Microsoft Mobile Device Center software. The date and time (hour, minute and second) settings for the MAJA Host and PC laptop were synchronized before data collection. To maintain continuous recording, power supply was required, via either battery or electricity.

Reading rates: The acceptance criterion for the reading was that **at least 95% of the crossing of bees should be recorded**. To ensure that this was possible, a performance check was conducted – before the system was fitted to the hive – by simulating honeybees crossing with tags glued onto small plastic or wooden sticks.
Protocol to control the performance of the RFID system and results of the RFID-reading rates 2018 and 2019 of the ring test group are presented in **Appendix 2**.

Fitting RFID equipment to the colonies: The first experimental hive was equipped with the RFID device at least **two days before the test**. For the other test colonies, a blank platform which mimicked the RFID system was placed at the hive entrance to allow the forager bees to familiarise themselves with the entrance style prior to fitting the RFID readers for the experiment.

Tag Batches: Pre-numbered 'Tag Kits' were used to tag the bees. Each kit contained RFID Tags which had previously been read and identified using a Pen reader to identify the UIDs of the tags in a particular kit. This information was stored in an excel spreadsheet. The kits were then allocated to a particular treatment group. This allowed the UIDs and hence the bees and kits to be tracked. Three to four batches of 10 to 15 tags (bees) were prepared per each test run and treatment.

4.3 Test item

Technical grade neonicotinoid active ingredient (a.i.) thiamethoxam
Supplier: Laboratories Dr. Ehrenstorfer-Schäfers, Augsburg
CAS number: 153719-23-4
Purity> 99.0%

All participating labs used technical grade thiamethoxam with the same batch number. Certificate of analysis 2018 and 2019 are proposed in **Appendix 3**.

4.4 Test design

Number of treatments:	1 control group and 3 test item groups
Number of bees labelled and exposed per treatment and run:	minimum of 30 bees ➔ 3 cages of 10 tested bees respectively for 30 bees tested (the cage is the experimental unit)
Number of test runs:	3, each one with a different colony

4.5 Preparation of the test item and test feeding solutions

The preparation of the test item and test feeding solutions is presented in **Appendix 4**.
Test item solutions: Stock solutions of the test item were prepared. These could be prepared in advance and stored in the refrigerator at 4 °C ± 4 °C for up to 5 days before the start of the test. Acetone was used as solvent. An untreated solvent control using acetone was prepared (purity ≥ 98%). The final volume of the test or control solutions in the sucrose feeding solution was 0.1% (v/v). It was previously shown, that up to a concentration of 1% acetone in sucrose solution had no significant effect on homing success of bees compared to bees receiving only untreated water (see final reports of the ring tests 2015 and 2016/2017).

Test feeding solutions: The test item and control solutions (acetone 0.1 % v/v) were administered in a sucrose feeding solution containing 30% (w/v) sucrose in demineralised water, corresponding to 30 g sucrose in 100 ml of demineralised water.

Test feeding solutions could be prepared up to 1 day before the test and stored at 4 °C ± 4 °C.

Test item, control and sucrose solutions were prepared fresh for each test run and stored in a deep freeze at –20°C ± 2°C after treatment of the bees for analytical determination of the actual treatment concentrations and dose per bee. To do so, the 3 treated test feeding solutions (≥ 5 ml of solution per sample) of each 3 test runs were forwarded to ITSAP laboratory before being sent to the French food safety agency (ANSES, Sophia Antipolis) for subsequent analyses.

4.6 Test cages

All laboratories used ventilated cages of an appropriate size for the number of captured foragers. For a good observation and handling of the tested bees, cages were designed appropriately, (either having transparent panels or being completely transparent) and could be opened easily to allow insertion and release of the bees.

4.7 Homing flight test procedure

Capture and preparation of "powdered" foragers
In the morning of the test day when the bees were actively foraging, returning foragers either with or without pollen were captured at the hive entrance.

In 2019, as far as possible, only pollen foragers were preferentially captured at the hive entrance. The percentage of pollen foragers on the total captured bees was recorded (**Appendix 5**).

Two equivalent methods were used to collect foragers:

1) One by one with entomological clamps
Bees were collected with entomological clamps (up to 300 individuals per group) and were placed in boxes (e.g. plastic food trays of 600 to 2000 cm^3 with for example 11 x 15 x 12 cm height). Bees were introduced in each box through a hole in the lid, which could be covered to prevent escape.

2) Collectively with an insect aspirator or other suitable system
Bees were collected with an insect aspirator or other system in containers (e.g. plastic bottles of 1000 cm^3 or boxes). Containers (bottles or boxes) were weighed empty and then weighed with the bees captured using a field precision balance (e.g. max 500 g, precision 0.1 g). The weight of bees was converted into the number of bees. To do so, **a group of 20 foragers of the experimental colony** were weighed to estimate the mean weight per bee.

A minimum of 600 returning bees carrying pollen were collected at the hive entrance and kept in boxes of max. 300 bees. Hydrophobic Powder (pink fluorescent pigments – T series, COLOREY SAS, France) was added in each box containing captured bees with a proportion of **0.3 mg per bee (e.g. 30 mg for 100 bees)**. Boxes/bottles were gently shaken in order to color the bees evenly. A preliminary acute toxicity study performed in 2016 showed that the pink hydrophobic powder alone or in combination with the tested test item doses did not lead to adverse effect on survival, sensitivity and natural behaviour of foragers compared to non-powdered bees exposed or not to the tested doses of thiamethoxam (see the report "Summary of the results of the international ring test 2016 and 2017").

Before or after being marked with the colored powder, collected foragers were transported to a release site located at 1 km (+/- 100 m) away from the experimental colonies. The release point was selected at a certain distance (e.g. 20 meters) from a landscape barrier (e.g. not in front of a hedge) for the bees to correctly fly away and return to the colony. Boxes/bottles were placed on a flat surface and opened to allow the bees to exit. If necessary, the bees were emptied out.

Recapture of the "powdered" foragers at the hive entrance
Recapturing of foragers: Colored bees returning to the hive were collected (on the flight board) up to a maximum of 2 hours following release (**Appendix 1**). Thus, bees captured had at least one homing experience to the hive from the release site. Bees were grouped into cages (up to 50 bees per cage) with food *ad libitum*. **Candy (**e.g. Apifonda®) **was used**. If necessary, water could also be provided once the bees were captured.

Number of foragers captured: A minimum of 140 foragers must be captured to obtain at least 30 foragers per treatment group.

Labelling and exposure in the laboratory
Feeding, starvation and labelling phase:
Captured foragers were transferred to the laboratory (holding a constant temperature of 23 ± 3°C during the entire experimental phase). Foragers were first provided with food *ad libitum* (candy: e.g. Apifonda®) for one hour to synchronize their dietary state. During this feeding period, cages were kept in dark conditions (e.g. half opened isolated box with a wet towel to avoid dehydration).Water could also be provided for the bees during this period.

After this period, the test started and the bees underwent a starvation phase of **90 mins duration (1.5 hours)**. During the starvation phase, the bees were transferred one by one from the cages to a holding cage (e.g. queen marking device, **Appendix 1**) where the bees were fixed and immobilised by a foam plunger without damage. Then, a RFID tag was glued with dental cement (e.g. Temposil®, Coltene) on the dorsally side of the thorax of each bee. Dental cement is non-corrosive and dries very quickly (less than 2 minutes).

During the labelling phase, the glue dispenser was placed in crushed ice when not in use to avoid the dental cement drying and blocking the tip. The labelling was performed without using anaesthetic on the bees.

The RFID tags were registered and allocated per treatment beforehand (cf. 4.3 RFID device). After labelling, the foragers were transferred in groups of 10 to 15 bees into cages (minimum of 3 cages of 10 bees per treatment). The cages with the RFID labelled bees were kept in the dark until the exposure phase.

Exposure phase: The test was conducted with three test item doses and one untreated control treatment (see table below). From 2018, tested sublethal doses were changed to take into account possible differences in sensitivity of the bees around the dose of 1-ng per bee. The two highest doses would correspond to a NOEL ('No Observed Effect Level') on mortality, 48-h after exposure.

Treatment	Test item doses
1	Untreated control (acetone 0.1 % (v/v)
2	0.33 ng per bee (acetone 0.1 % (v/v)
3	1 ng per bee(acetone 0.1 % (v/v)
4	1.5 ng per bee (acetone 0.1 % (v/v)

Exposure procedure: The honeybees were exposed by feeding them with 20 µl per honeybee (200 µl per group of 10 bees) of the 30% (w/v) sucrose solution containing the test item at different concentrations and the control solution (treatments). The volume of the treatments was provided using a feeder system enabling contact with the food only through the mouth parts (e.g. the tip of a micropipette bevelled). The bees in each cage shared the feeding solution via trophallaxis.

Exposure conditions: The minimum exposure duration was 1 hour in dark conditions for all treatments to limit the stress. If bees in some cages did not consume all the provided treatment solution within one hour, the exposure phase was prolonged for all bees and treatment groups for a maximum of 30 min or until all bees had consumed the sucrose solution within this time (max. exposure phase 1.5 hours). The start and end time of exposure duration was recorded.
Remaining treatment solution in any of the test cage feeders after the max. exposure phase of 1.5 hours, was measured by weighing the feeders in order to calculate the actual volume and dose consumed per bee. To do so, volume of sucrose solution prepared was weighed each time before the exposure phase for density determination.

Post-exposure: After exposure phase (60 min. (minimum) to 90 min. (maximum)), honeybees underwent an additional 40 minutes starvation period in the dark. During post-exposure starvation, cages were kept in an isolation box (e.g. cooler) including a wet towel in order to avoid dry up (the lid should be half-open).

Mortality and lost tags: During the release phase, any dead bee and lost tags were collected, identified (thanks to the UID of the tag) and recorded. They were excluded from any homing performance calculations. As a validity criterion, mean mortality of control bees over all replicates should not to exceed 15%

Honeybees release
Transport: The treated honeybees were transported **to the same release site as** at the first time after coloring at 1 km (+/- 100 m) from the colony
Temperature and humidity levels during transport were maintained to ensure their safe keeping, particularly if the release place is far away from the laboratory (transport of the bee cages in cool boxes containing a damp cloth, in a box incubator…)

Before release: the cages representing the tested treatments were put on a flat surface at least a few centimeters off the ground, and then opened simultaneously. If necessary, the bees were emptied out.

At release time, weather conditions should be favourable for foraging activity (wind below 5 of Beaufort scale, temperatures of at least 15°C and no rain).
Release start and end time (hour and minutes), local weather conditions (temperature and hygrometry (%)) were recorded during the release phase. Cloud cover and wind strength were also estimated (**Appendix 6)**.

Release time and homing flight recording: release time was **at least two hours** before sunset to allow foragers enough time to fly back to the hive. The homing flight of tagged bees was recorded during 24-h after release.

4.8 Test Schedule

Bee powdering, capture, labelling, exposure and release phases for the test took place over one day.

The homing flight recording of the labelled foragers started immediately after the release and continued for 24 hours. This 24h-recording period was sufficient for assessing the homing success of released bees (Henry et al. 2012, homing flight ring test results 2015 to 2019).

4.9 Assessment

The data recorded with RFID readers for the bees returning to the hive: Following raw data were recorded in electronic form (MAJA Host storage system equipped with the appropriate software via PC connected to the host): the UID of the tag, the reader number and the reading time (date, hour, minute and second). Data were collected for 24 hours after the release.

The weather conditions: temperature (T°C) and hygrometry (%) were recorded at least once per hour using a data logger placed under the tested hive with RFID system. Rainfall (mm) per day was also recorded at the same place using a rain gauge.

Landscape: a map of the area with location of the tested colonies and release site labelled on the map (e.g. Google Earth map) was established. The GPS coordinates of the colonies location and of the release point were given or indicated on the map. From the map, landmarks (roads, hedges, buildings, rivers…) that the bees can cross when returning to the hive were counted as a measure of landscape complexity. Indeed, Henry et al. 2014 showed that exposed bees had lower homing performances with higher number of landmarks, that is, when landscape is more complex. The counting was performed on a trajectory more or less linear from the release site to the hive (deviation of about 20°on both sides of the release point) according to the results of Fisher et al. (2014).

After the ring test 2018, **a questionnaire** was proposed to each lab to try to identify and solve critical points and problems with the homing flight method.

4.10 Summary of the test procedure changes in 2018 and 2019

	2018	**2019**
Type of foragers first collected (before being colored)	All returning foragers to the hive (pollen or nectar)	Focus as far as possible on pollen collectors returning to the hive
Pre-exposure starvation	1h30	1h30
Exposure phase	1h00 (1h30 maximum)	1h00 (1h30 maximum)
Post-exposure starvation	40 mins	40 mins
Feeding *ad libitum* before release	No	No

4.11 Results presentation and statistical analysis

- **Homing success and homing duration**

After labelling and before release in the field, the number of dead bees was used to calculate a mortality rate per treatment for each test run.

Homing performance was characterised by two variables:

- The homing flight success (**main variable**), which is a binomial variable with a value of 1 if the honeybee returns to the hive and is recorded over the 24-hours period, or 0 if it does not return.
- The homing time 24 hours after release (**secondary variable**), which is a quantitative variable. For each honeybee, it is defined as the time between the release and the first recording when passing the readers (entering the hive).

Data files organisation and statistical analysis were performed using the software R version 3.3.1 (R Development Core Team, 2016). Homing success and its duration was determined from three files: **1.** honeybee identification and treatment allocation (with UID of the tags), **2.** information at the release place (date, hour and minutes of release), **3.** RFID recording at the hive entrance. The three data sets were used to provide one raw data file per identified honeybee and treatment where homing time was expressed in minutes. During the 24 hours of RFID recording, a honeybee can be recorded several times when it passes the RFID reader (in or out the hive) for foraging activities. Therefore, several data points were recorded and can be calculated for the same bee. We only used the shortest homing time per bee, which corresponds to the first recording of the tagged bees at the hive after release. A bee which didn`t return to the hive after release was missing in the raw data, and was identified due to the missing UID in the raw data when compared to the registered UIDs before release.

One raw data file was created per run and all three runs pooled for the data analysis. Data from the three test runs were pooled to maximize the total number of bees per treatment (total of ≥ 90 bees labelled with a RFID tag) for the homing test analysis including data structuration and statistical treatments (Henry et al. 2012). The results per run or for the pooled data are presented as cumulative homing probability of the bees to the hive over the 24-hours period per test item treatment and control group. Homing duration was illustrated as boxplots (medians, quartiles).

From the results of the three test runs (pooled data), the bee homing rates back to the hive obtained over the 24-hour period for each treatment were compared using a Chi^2 test ($P < 0.05$). An adjusted significance threshold was applied for paired comparisons with Bonferroni method. Concerning homing duration, data normality and homogeneity of variance were first tested with a Shapiro-Wilk test and a Bartlett test respectively ($P > 0.05$). As data didn't show normal distribution and/or variance homogeneity, homing durations obtained were compared between treatments using a non-parametric Kruskal-Wallis test ($P < 0.05$) followed by a Mann-Whitney test for paired comparisons. An adjusted significance threshold was also applied for paired comparisons with Bonferroni method.

From the test data analysis, we determined a 'No Observed Effect Dose' (NOED) on the homing flight. The NOED was expressed as **ng test item per honeybee**.

- **Analysis of homing results variability**

In order to assess the effects of different factors on the homing performance ($P< 0.05$), we used generalized linear mixed-effects models (GLMMs) with a logit link function using the R package lme4 (Bates et al., 2018). We considered test runs' data of all the labs. The homing flight was considered as a binary response variable (0 = no return, 1 = return during the 24 hours of recording). The identity of experimental colonies and of the release sites were included as random variables. The real exposure dose was introduced as a fixed, quantitative, explanatory variable. Additional explanatory variables were temperature (punctual temperature at the release time), Varroa mite infestation of the colonies (number of varroa per 100 bees) and landscape (number of landmarks that bees can cross during the returning to the colony as an indicator of landscape complexity).
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 of a simpler model based on the Akaikee information criterion (AIC), gives the probability that the model 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 with 95% chance of including the most parsimonious combination of explanatory variables.

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 of size of effect and were comparable among each other. Data for Varroa and landscape variable were log10-transformed.

4.12 Validity criteria of the study

Validity criteria were considered as:

- Mortality rate in control bees after exposure and before release ≤ 15 % for each test run
- The minimum and acceptable homing success rate of control bees for each test run of at least 60% over the 24 hours period.

5-RESULTS AND DISCUSSION

Eight laboratories out of 10 could perform the test in 2018 and 2019. 24 and 23 test runs were conducted in 2018 and 2019, respectively. Administered volumes of control and treated sucrose solution to the caged bees during exposure phase were totally consumed each time, for each treatment and run.

5.1 Mortality before release

Bee mortality was recorded from the end of the exposure phase until the release phase in the field.

Tables 1 to 6 present numbers of foragers labelled and released in 2018 and 2019. Bees not released include dead bees and/or bees that lost their tags. Mortality before release was generally low and met the validity criterion (**dead bees ≤ 15 %**) for control bees (except for one run in 2019) but also for 0.33 and 1-ng exposed bees.
Some lethal effects (**dead bees > 15 %**) could punctually appear especially for the bees exposed to the highest dose of 1.5 ng per bee. For 1.5 ng exposed bees, run 1 of Lab1, run 2 of Lab 5, run 3 of Lab 2 are concerned in 2018; run 1 of Lab 8, run 2 of Lab 7, run 3 of Lab 6 and Lab 7 in **2019** (Figures 2 to 7).

Table 1: Number of labelled (LB) and released bees (RB) for the test run 1 in 2018

Lab	Bee race	Nb of bees	Control	0.33 ng/bee	1 ng/bee	1.5 ng/bee
1	Carnica	LB	30	30	30	30
		RB	28	28	27	23
2	Ligustica	LB	30	30	30	30
		RB	30	30	30	30
3	Carnica	LB	30	30	30	30
		RB	30	30	20	30
4	Buckfast	LB	30	30	30	30
		RB	29	28	26	24
5	Ligustica	LB	30	30	30	30
		RB	25	27	28	27
6	Black x Buckfast	LB	40	40	40	40
		RB	40	39	39	39
7	Buckfast	LB	30	30	30	30
		RB	29	29	30	28
8	Carnica	LB	42	42	42	42
		RB	41	38	42	37

In red, results for which dead bees > 15 %

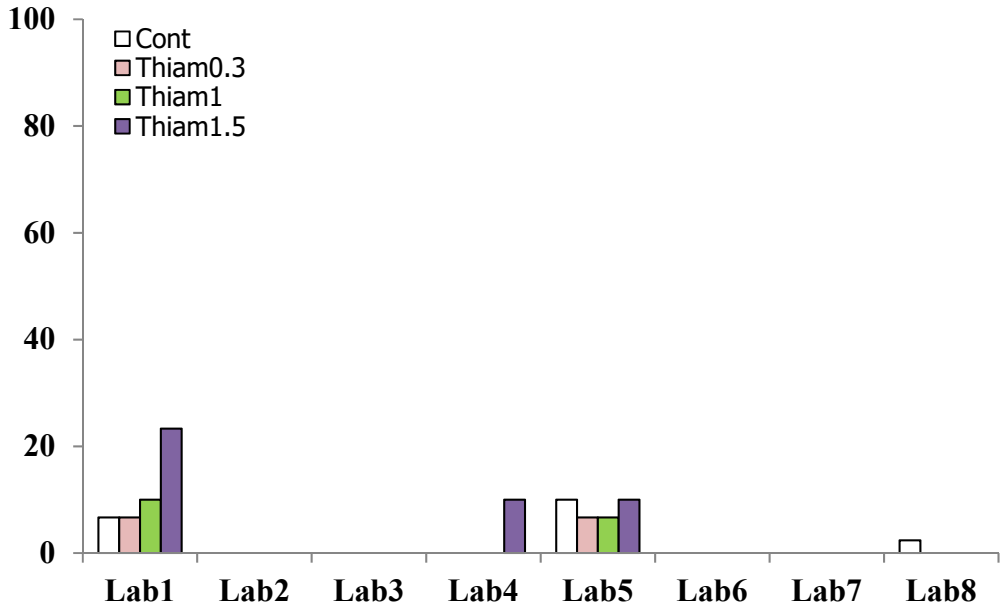


Figure 2: Mortality rate (%) before release for the test run 1 in 2018

Table 2: Number of labelled (LB) and released bees (RB) for the test run 2 in 2018

Lab	Bee race	Nb of bees	Control	0.33 ng/bee	1 ng/bee	1.5 ng/bee
1	Carnica	LB	30	30	30	30
		RB	28	28	30	30
2	Ligustica	LB	30	30	30	30
		RB	29	29	30	30
3	Carnica	LB	40	40	40	40
		RB	39	38	38	40
4	Buckfast	LB	30	30	30	30
		RB	30	30	28	30
5	Ligustica	LB	30	30	30	30
		RB	22	17	23	20
6	Black x Buckfast	LB	40	40	40	40
		RB	39	39	40	34
7	Buckfast	LB	30	30	30	30
		RB	30	30	30	29
8	Carnica	LB	42	42	42	42
		RB	41	40	42	40

In red, results for which dead bees > 15 %

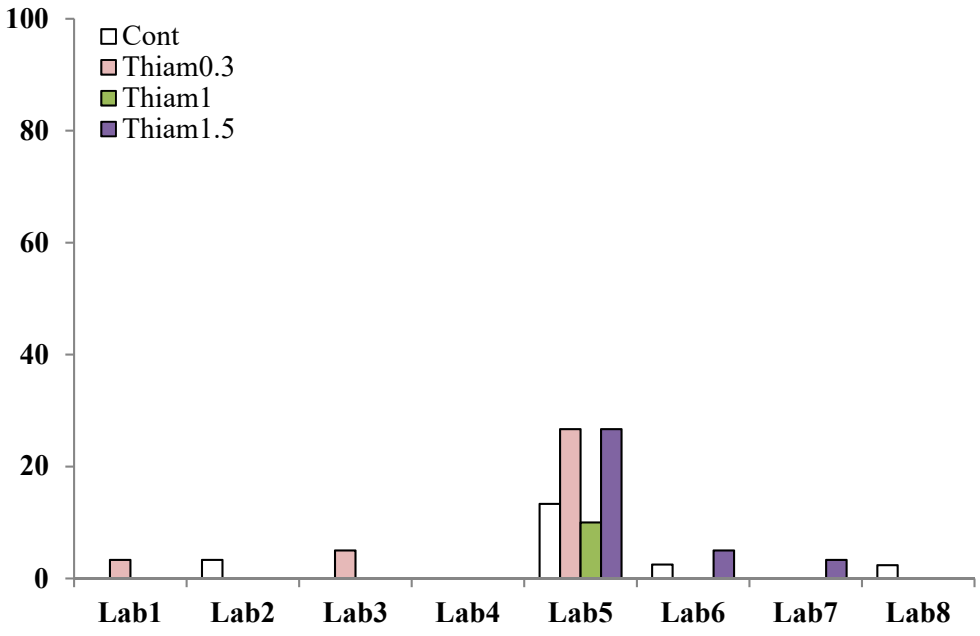


Figure 3: Mortality rate (%) before release for the test run 2 in 2018

Table 3: Number of labelled (LB) and released bees (RB) for the test run 3 in 2018

Lab	Bee race	Nb of bees	Control	0.33 ng/bee	1 ng/bee	1.5 ng/bee
1	Carnica	LB	30	30	30	30
		RB	30	30	30	28
2	Ligustica	LB	30	30	30	30
		RB	30	30	30	12
3	Carnica	LB	40	40	40	40
		RB	37	37	40	37
4	Buckfast	LB	30	30	30	30
		RB	30	29	24	22
5	Ligustica	LB	30	30	30	30
		RB	25	29	23	28
6	Black x Buckfast	LB	40	40	40	40
		RB	37	30	35	33
7	Buckfast	LB	30	30	30	30
		RB	30	30	30	28
8	Carnica	LB	42	42	42	42
		RB	40	39	41	41

In red, results for which dead bees > 15 %

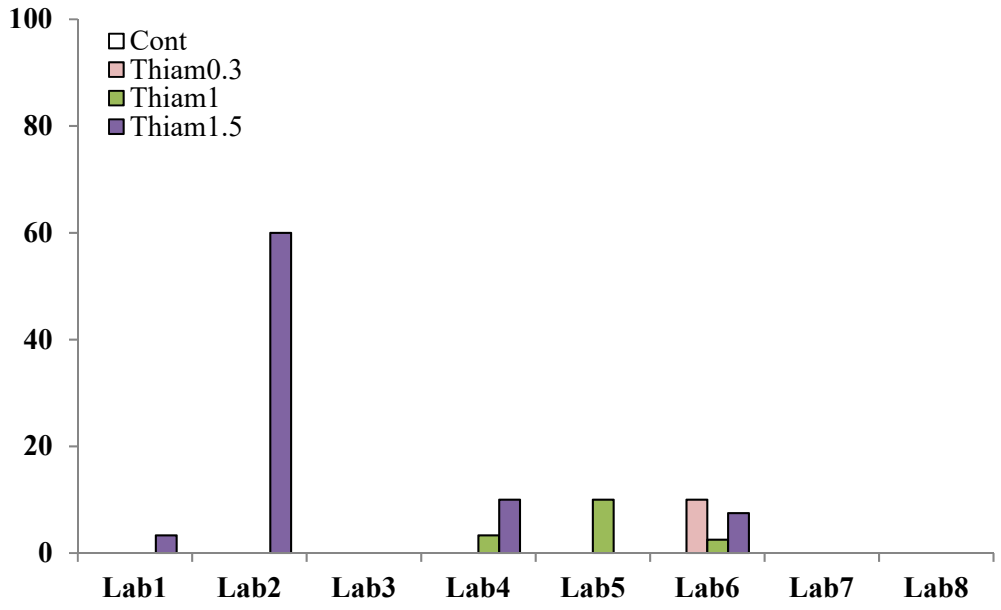


Figure 4: Mortality rate (%) before release for the test run 3 in 2018

Table 4: Number of labelled (LB) and released bees (RB) for the test run 1 in 2019

Lab	Bee race	Nb of bees	Control	0.33 ng/bee	1 ng/bee	1.5 ng/bee
1	Buckfast	LB	30	30	30	30
		RB	27	28	25	27
2	Ligustica	LB	30	30	30	30
		RB	30	30	30	25
3	Carnica	LB	30	30	30	30
		RB	28	30	30	28
4	Carnica	LB	40	40	40	40
		RB	39	37	38	39
5	Black x Buckfast	LB	40	40	40	40
		RB	39	39	39	39
6	Buckfast	LB	30	30	30	30
		RB	29	30	30	29
7	Ligustica	LB	30	30	30	30
		RB	25	25	23	24
8	Carnica x Buckfast	LB	40	40	40	40
		RB	32	36	32	33

In red, results for which dead bees > 15 %

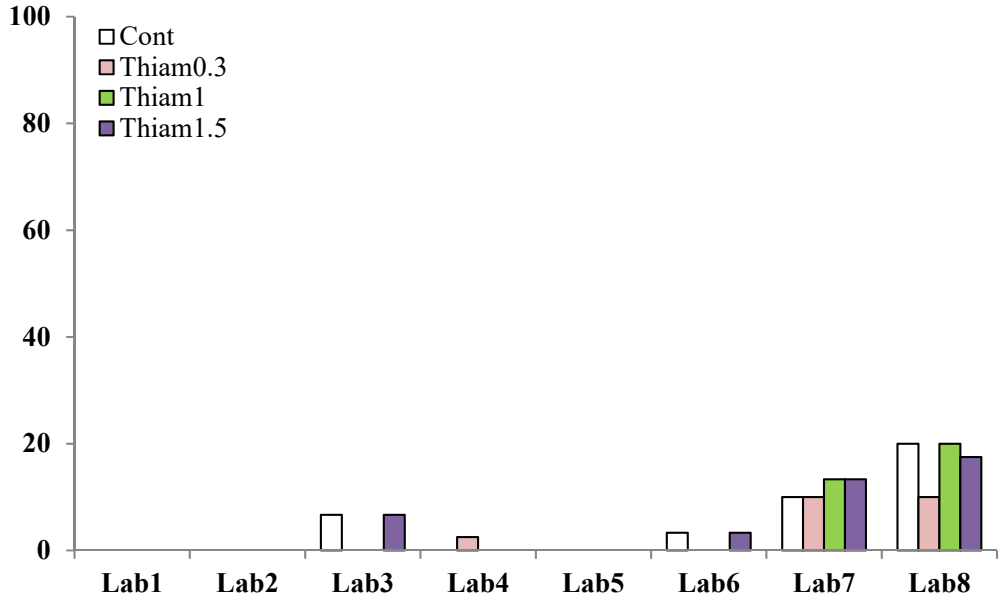


Figure 5: Mortality rate (%) before release for the test run 1 in 2019

Table 5: Number of labelled (LB) and released bees (RB) for the test run 2 in 2019

Lab	Bee race	Nb of bees	Control	0.33 ng/bee	1 ng/bee	1.5 ng/bee
1	Buckfast	LB	30	30	30	30
		RB	29	28	29	25
2	Ligustica	LB	30	30	30	30
		RB	29	30	30	26
3	Carnica	LB	30	30	30	30
		RB	27	25	24	27
4	Carnica	LB	40	40	40	40
		RB	40	39	37	40
5	Black x Buckfast	LB	40	40	40	40
		RB	38	37	38	37
6	Buckfast	LB	30	30	30	30
		RB	30	30	30	28
7	Ligustica	LB	30	30	30	30
		RB	24	24	23	21
8	Carnica x Buckfast	LB	40	40	40	40
		RB	35	34	32	30

In red, results for which dead bees > 15 %

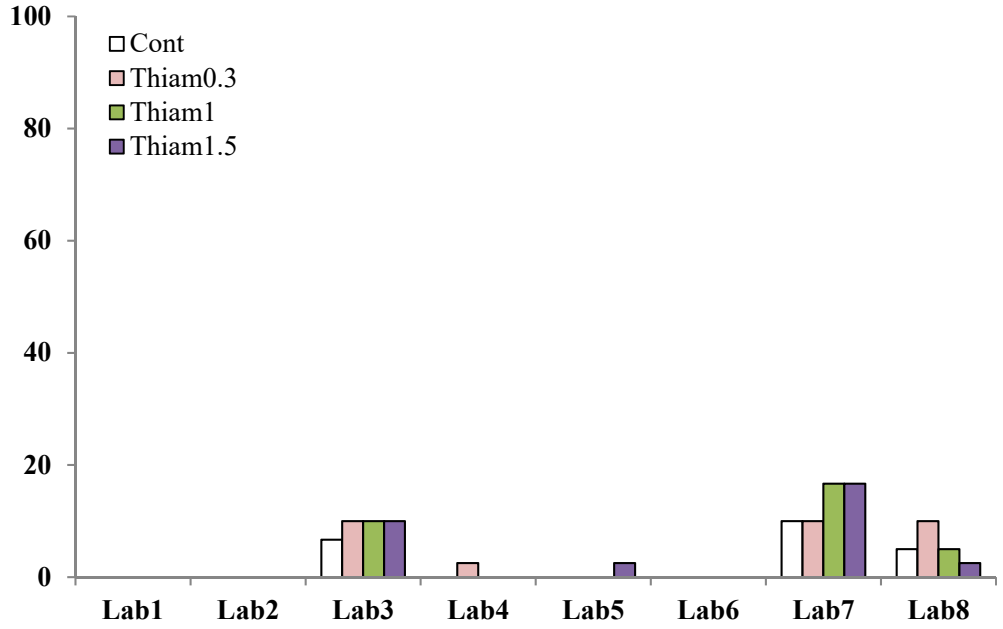


Figure 6: Mortality rate (%) before release for the test run 2 in 2019

Table 6: Number of labelled (LB) and released bees (RB) for the test run 3 in 2019

Lab	Bee race	Nb of bees	Control	0.33 ng/bee	1 ng/bee	1.5 ng/bee
1	Buckfast	LB	30	30	30	30
		RB	28	29	27	27
2	Ligustica	LB	30	30	30	30
		RB	30	28	30	29
3	Carnica	LB	30	30	30	30
		RB	30	27	27	27
5	Black x Buckfast	LB	40	40	40	40
		RB	37	38	38	40
6	Buckfast	LB	30	30	30	30
		RB	29	29	30	24
7	Ligustica	LB	30	30	30	30
		RB	28	29	21	13
8	Carnica x Buckfast	LB	40	40	40	40
		RB	34	30	31	33

In red, results for which dead bees > 15 %

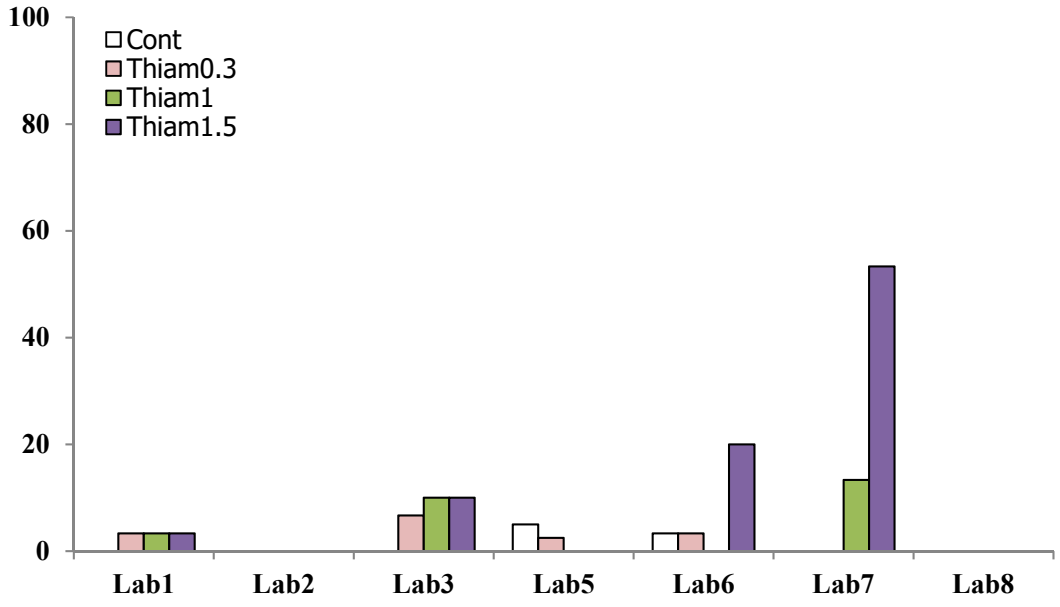


Figure 7: Mortality rate (%) before release for the test run 3 in 2019

Summary of the mortality for the five years of ring test

Over the five years of ring test, control bees' mortality ranked from 0 to 15 % in 96.8 % of the test runs (n = 125 values). Except for 4 test runs, **the validity criterion of ≤ 15% of dead bees** was met since 2015 (Table 7).

Table 7: Mortality in control bees ranked according to mortality rate classes (%) for 125 test runs performed over the five years of ring test (n= 125 mortality values for control groups)*

	[0-5[	[5-10[	[10-15[	[15-20[	[20-25[	[25-30[	[30-35[
2015	**18**	**3**	**1**	0	0	0	0
2016	**21**	**6**	**2**	0	1	0	0
2017	**21**	**2**	**1**	1	0	0	0
2018	**21**	**0**	**3**	0	0	0	0
2019	**16**	**4**	**2**	0	1	0	0

** One mortality rate ≥ 35% in 2016*

Considering treated groups, mortality decreased to ≤ 15 % in the majority of cases before release for the last three ring test years (Table 8). This could be explained by the gained experience with the manipulation of the bees in the laboratory. Then, **mortality is for instance ≤ 15 % in 94.4 %** of the cases **in 2018** (n= 72 values for all treated groups) and in **91.3 %** of the cases **in 2019** (n= 69 values for all treated groups). For these two years, only 10 values were above 15 % of mortality, and 7 of them were for the 1.5-ng exposed bees. A validity criterion of **≤ 15% dead bees** could be considered not only for control bees but for exposed bees too.

Table 8: Mortality in exposed bees ranked according to mortality rates (%) classes for 125 test runs performed over the five years of ring test (n= 375 mortality values for all treated groups)*.

	[0-5[	[5-10[	[10-15[	[15-20[	[20-25[	[25-30[	[30-35[
2015	**47**	**10**	**3**	**4**	1	1	0
2016	**68**	**9**	**6**	3	2	2	2
2017	**63**	**7**	**3**	0	1	0	0
2018	**55**	**6**	**7**	0	0	2	1
2019	**46**	**3**	**14**	3	2	0	0

**Four mortality rates ≥ 35%: one in 2016 for the 0.1-ng exposed bees; one in 2017 for the 1-ng exposed bees; one in 2018 and one in 2019 for the 1.5-ng exposed bees*

5.2 Homing performance in control bees

Summary of the homing performances in control bees for the five years of ring test

For a majority of test runs, homing performance in control bees ranked from 60 to 100 %. It also progressed from 70 to 100 % in 2017 and 2019 (Table 9).

According to the ring test results the **minimum and acceptable homing performances in control bees vary from 60 to 70 %, which might be used as a validity criterion**.

Table 9: Homing performances in control bees ranked according to homing rate (%) classes for 125 test runs performed over the five years of ring test.

	2015	2016	2017	2018	2019
Homing rate classes for control bees (%)	**Nb of tests**	**Nb of tests**	**Nb of tests**	**Nb of tests**	**Nb of tests**
[0-10[	0	0	0	2	1
[10-20[	0	1	0	0	0
[20-30[	0	1	0	1	1
[30-40[	2	0	0	0	2
[40-50[	0	0	0	2	2
[50-60[	3	4	3	2	1
[60-70[	7	5	**3**	5	**2**
[70-80[	1	**5**	4	**4**	6
[80-90[	3	**5**	**11**	**3**	**5**
[90-100[	6	**10**	**4**	**5**	**3**
TOTAL	**22**	**31**	**25**	**24**	**23**

The percentage of valid test runs was considered with the minimum homing performance in control bees ≥ of 60, 70 or 80 % (Figure 8). Based on the results of the five ring test years, a minimum homing performance of 80% cannot be accepted because too many test runs would be invalid. Best results were obtained in 2017. However, in 2016 and 2017, bees were fed *ad libitum* before release. Feeding increased the stomach content and the effects of the insecticide (highest dose tested) could not be detected anymore due to possible dilution of the remaining stomach content origin of the exposure phase (see "summary of the results of the international ring test 2016 and 2017"). **One compromise are the results obtained in 2019**. Indeed, only 69.6 % test runs met the category ≥ 60 % (two labs performed 3 invalid test runs below 60 % of homing performances for the control bees). But when considering the category ≥ 70 %, percentage of valid test runs are relatively similar to the category ≥ 60 % with 65.2 % test runs. Only one test run was between 60 and 70 % considering the homing performance in control bees, whereas all others were ≥ 70%.

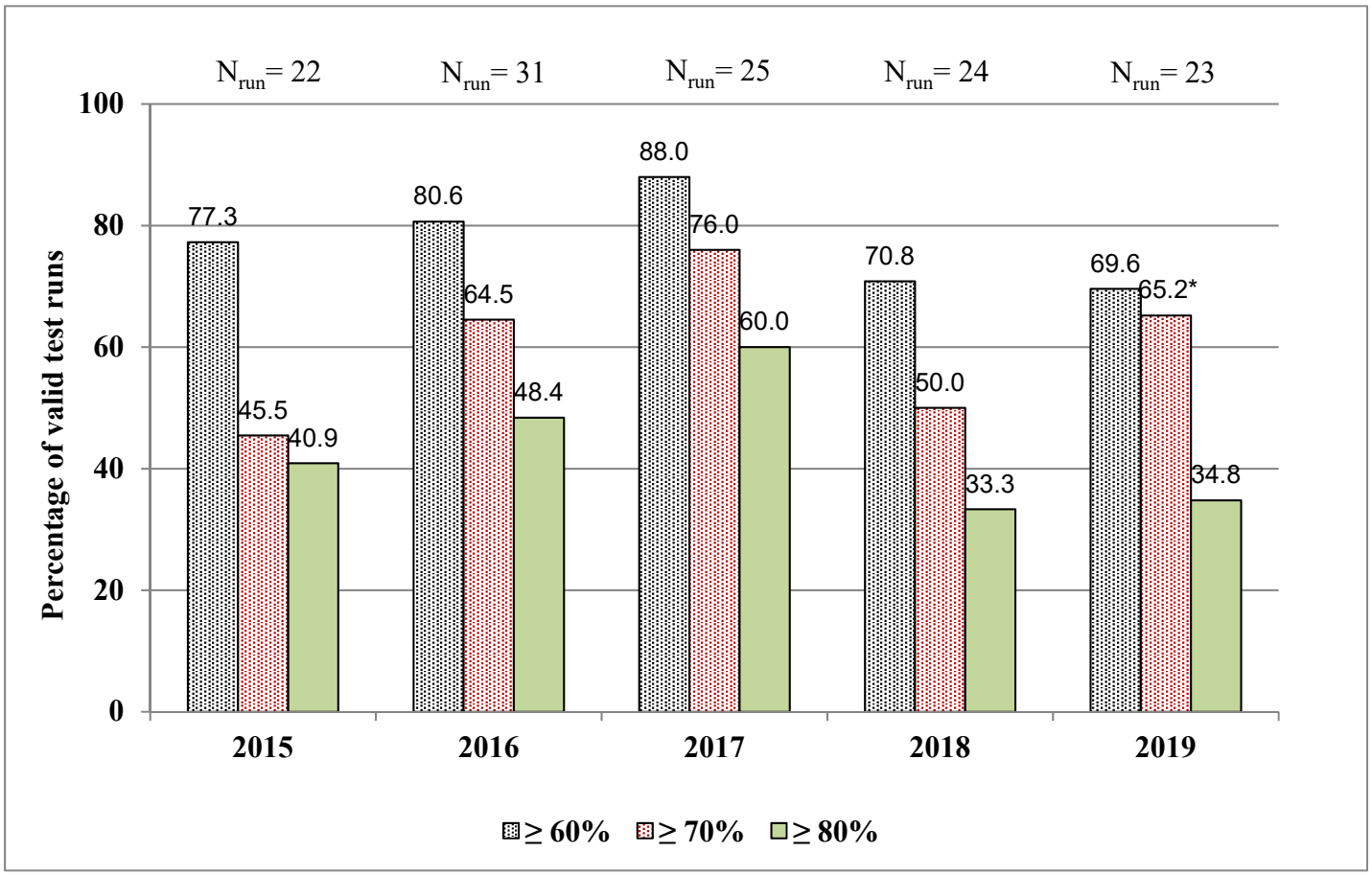


Figure 8: Percentage of valid test runs when the minimum accepted homing performances in control bees is ≥ 60, 70 or 80 %. * *One homing rate at 69 % considered in the category ≥ 70 %.*

Comparison with "natural" loss of foragers in field conditions (F. Requier, com. pers.)

Minimum and acceptable homing performances of control bees were compared to the natural" loss rates of "free-ranging foragers under field conditions. To do so, we considered a R&D study previously performed with the RFID system from April to September 2015 in the North-West of France. Groups of emerging bees from three colonies were tagged each month during the period (total: 2100 emerging bees tagged). Data were obtained for 80 % of the labelled bees. With continuous recording, bees' activity (e.g. entry and exit from the hive) could be followed each day from adult emergence to the death (no more RFID recording). Median foraging age (or median age of first foraging) was determined and calculated for each group of bees. Then, daily survival rate (%) was determined for each group of foragers and dates, from the age of first foraging to the end (when less than 10 tagged remaining bees were recorded). This survival rate (returned bees back to the colony) was calculated as number of bees recorded in the evening on day (D) / number of bees recorded in the evening the day before (D-1). All the calculated daily survival rates (%) of foragers were distributed according to Figure 9.

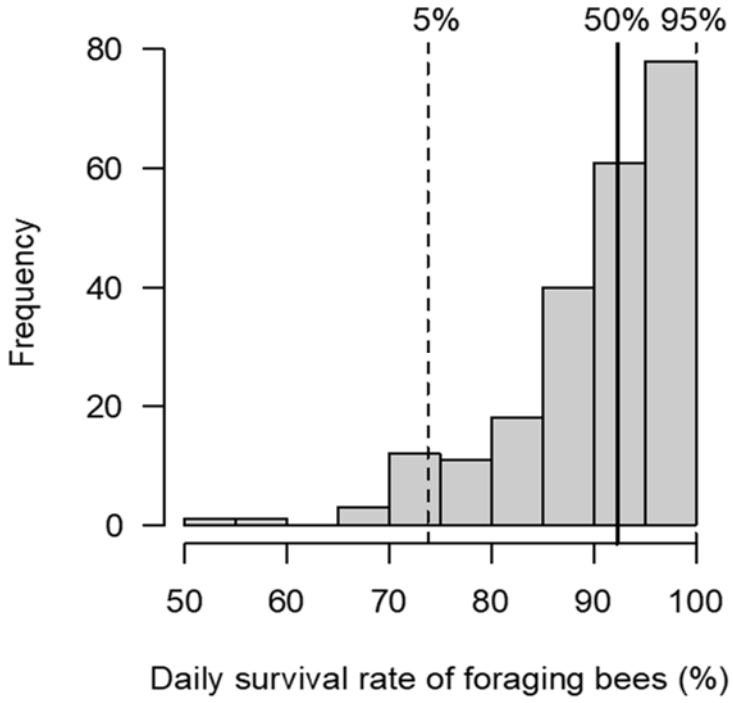


Figure 9: Ranking of all daily survival rate (%) of free-ranging foragers under field conditions. All recorded daily survival rates of foragers ranked between 50 and 100 %.

Figure 9 shows that the median daily survival rate is > 90 % but the distribution begins at 70 % (5% of the results). Then, this study would support a maximum daily foragers' loss of 30 %. This is comparable to the minimum and acceptable level of homing failure of 30 to 40 % considered for the control foragers (minimum and acceptable homing performances of 60 to 70 %).

5.2 Homing success per treatment and run

Homing performances per treatment and run were calculated 24 hours after release.

For a majority of test runs, results showed lower homing performances for the bees exposed to the highest doses (1 and 1.5-ng exposed bees) compared to control bees or 0.3 ng-exposed bees for both years (Tables 10 and 11; Figure 10).

When comparing control and 1-ng exposed bees, 4 valid runs[1] out of 17 (23.5 %) in 2018 and 2 valid runs on 16 (12.5 %) in 2019 showed low (≤ 10 %) or no differences in homing performances. **When comparing control and 1.5-ng exposed bees**, 2 valid runs out of 17 (11.8 %) in 2018 and 2 runs on 16 (12.5 %) in 2019 showed low (≤ 10 %) or no differences in homing performances (Table 10 and 11).

[1] Test run is considered valid when homing performances of control bees ≥ 60 %

Table 10: Results of homing probabilities per test run and laboratory in 2018

Lab	Run	Control	Thiam. 0.3ng/bee	Thiam. 1 ng/bee	Thiam. 1.5 ng/bee
1	1	0.750	0.893	0.556	0.000
	2	0.786	0.607	0.633	0.167
	3	0.900	0.867	0.600	0.321
2	1	0.933	0.700	0.433	0.333
	2	0.828	0.931	0.733	0.567
	3	0.633	0.600	0.367	0.333
3	*1	0.533	0.367	0.450	0.000
	*2	0.461	0.342	0.474	0.050
	*3	0.216	0.351	0.275	0.243
4	1	**0.621**	**0.821**	**0.769**	**0.542**
	2	0.833	0.700	0.607	0.500
	3	0.667	0.414	0.125	0.091
5	*1	0.040	0.259	0.179	0.074
	2	0.682	0.529	0.217	0.000
	*3	0.560	0.724	0.522	0.143
6	1	0.650	0.667	0.487	0.256
	2	0.744	0.744	0.075	0.176
	3	0.784	0.767	0.514	0.333
7	*1	0.414	0.655	0.367	0.321
	*2	0.067	0.100	0.600	0.241
	3	0.867	0.567	0.433	0.643
8	1	**0.902**	**0.895**	**0.952**	**0.622**
	2	**0.927**	**0.950**	**0.857**	**0.775**
	3	**0.900**	**1.000**	**0.976**	**0.829**

* Invalid test runs (homing success of control bees < 60%)

Runs with no or low differences in homing success between 1ng-exposed bees and control bees (≤ 10 % differences in homing performances) or homing success of 1ng-bees higher than control bees.

Runs with no or low differences in homing success between 1.5 ng-exposed bees and control bees (≤ 10 % differences in homing performances) or homing success of 1.5 ng-bees higher than control bees.

Table 11: Results of homing probabilities per test run and laboratory in 2019

Lab	Run	Control	Thiam. 0.3ng/bee	Thiam. 1 ng/bee	Thiam. 1.5 ng/bee
1	**1**	0.741	0.786	0.560	0.333
	2	0.793	0.893	0.750	0.080
	3	0.893	0.828	0.778	0.407
2	**1**	0.600	0.533	0.300	0.040
	2	0.724	0.633	0.433	0.154
	3	0.833	0.750	0.667	0.724
3	**1**	0.821	0.900	0.500	0.286
	2	0.704	0.720	0.542	0.185
	3	0.967	0.926	0.741	0.296
4	**1**	0.718	0.595	0.474	0.179
	2	0.825	0.923	0.730	0.800
5	**1**	1.000	1.000	0.795	0.846
	2	0.974	0.919	0.684	0.595
	3	0.838	0.789	0.711	0.500
6	***1**	0.345	0.367	0.267	0.207
	2	0.767	0.800	0.267	0.250
	3	0.690	0.621	0.567	0.125
7	***1**	0.520	0.400	0.000	0.000
	***2**	0.417	0.500	0.000	0.000
	***3**	0.357	0.207	0.000	0.000
8	***1**	0.060	0.111	0.125	0.121
	***2**	0.257	0.147	0.031	0.000
	***3**	0.471	0.100	0.065	0.030

* Invalid test runs (homing success of control bees < 60%)

Runs with no or low differences in homing success between 1ng-exposed bees and control bees (≤ 10 % differences in homing performances) or homing success of 1ng-bees higher than control bees.

Runs with no or low differences in homing success between 1.5 ng-exposed bees and control bees (≤ 10 % differences in homing performances) or homing success of 1.5 ng-bees higher than control bees.

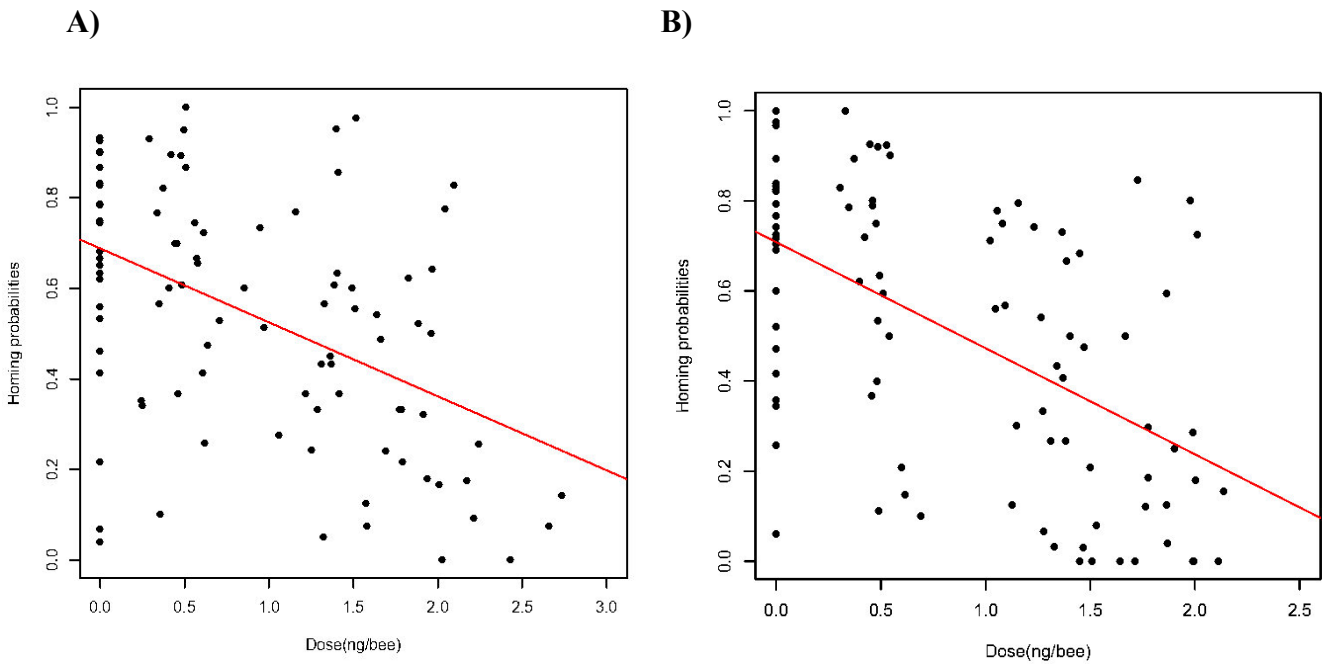


Figure 10: Relationships between homing probabilities of the foragers per test run 24 hours after release and the actual doses of exposure (ng per bee) from analyses. A red regression line was added. In 2018, two values were not included because of problems with the analytics (high and abnormal real values > 3 ng/bee, see part 5.4). **A) results obtained in 2018**, **B) results obtained in 2019**.

5.3 Climatic conditions during the tests

Climatic conditions recorded during the homing flight tests 24h-after the bees' release are presented in Tables 12 and 13 for 2018 and 2019 respectively.

In 2018, a summer heat wave occurred in July/August with difficulties to perform the test for some labs (e.g. Lab 3; Lab 4 especially for run 3). One lab couldn't perform any test at all because of problems with temperature. For Lab 3, the test was planned earlier in the season (May to July) but problems with tags delivery obliged to delay the test to August. Then for this lab, higher temperatures encountered have played a role in the lower homing performance as they faced in 2018.
For other labs, temperature was not a limiting factor (questionnaires 2018). In majority, climatic conditions alone do not explain invalid test runs (homing performances in control bees < 60 %) obtained in 2018 and 2019. When rainfall was recorded the day of release, it was in general a few hours after the bees' release and the impact was limited. Based from the ring test experience, it is now well known that the great majority of bees (familiar with their environment) will return to the hive within 2 hours after release (> 90 % of the bees recorded 24-h after release).

As a whole, the homing test performance rely on favourable climate according to geographic conditions (no temperatures too low (>15 °C) but also not too high and abnormal temperature for the region) associated with blooming flowers or crops in the surroundings for foraging activities. As far as possible, the best period for the test performance is spring/beginning of summer when the colonies develop and food resources are available (high nectar and pollen flow). If nice weather conditions are experienced in the late season (e.g. August/September) with blooming flowers/crops , test can be performed but making sure that Varroa pressure is low (see part **5.7**).

Table 12: Mean climatic conditions recorded during 24 hours after bees' release for each laboratory and run in 2018

Lab	Run	Mean temperature (°C)	Mean hygrometry (%)	Mean rainfall (mm)
1	1	22.97	57.22	0
	2	25.15	72.60	0
	3	23.9	86.19	0
2	1	25.31	62.10	0
	2	24.04	67.52	3[a]
	3	19.66	63.24	0
3	*1	27.19	52.53	0
	*2	24.25	55.01	0
	*3	19.72	59.56	0
4	1	29.74	57.54	0
	2	29.18	44.66	0
	3	31.66	52.26	0
5	*1	22.43	75.36	0.18
	2	26.76	62.44	0
	*3	26.17	55.84	0
6	1	18.95	78.04	0
	2	23.25	69.12	0
	3	17.20	62.72	0
7	*1	20.22	51.88	0
	*2	-	-	0
	3	22.19	-	0
8	1	21.98	87.08	13[b]
	2	19.80	54.84	0
	3	23.38	64.08	0

* Invalid test runs because of low homing success of control bees (< 60%)
[a] Storm and heavy rain after release at night (22.00 pm)
[b] Heavy rain one hour after release (duration 1h); some rain the following day

Table 13: Mean climatic conditions recorded during 24 hours after bees' release for each laboratory and run in 2019

Lab	Run	Mean temperature (°C)	Mean hygrometry (%)	Mean rainfall (mm)
1	**1**	20.52	47.92	0
	2	23.38	46.48	0
	3	25.88	58.16	0
2	**1**	18.34	64.46	0
	2	22.42	67.08	0
	3	24.60	44.80	0
3[a]	**1**	-	-	0
	2	-	-	0.7
	3	-	-	0
4	**1**	19.02	55.44	0.9
	2	19.68	68.46	0.4
5	**1**	21.74	64.27	0
	2	18.51	57.96	0
	3	21.27	70.77	0
6	***1**	18.78	82.98	0
	2	20.24	72.32	0
	3	22.62	57.26	0
7	***1**	29.87	57.45	0
	***2**	27.40	59.58	0
	***3**	28.52	61.36	0
8	***1**	22.12	49.31	0
	***2**	19.18	74.01	2.6[b]
	***3**	24.33	63.70	0

***** Invalid test runs because of low homing success of control bees (< 60%)

[a] No data for Lab 3 because of unforseen problems (no battery left for the data logger)

[b] Rainfall more than 3 hours after release (20.20 pm) for a 2.5-h duration

5.4 Analyses of the test item solutions

The test feeding solutions were analysed by the French Food Safety Agency (ANSES) laboratory (Sophia Antipolis) using the liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) technique to detect real concentrations of thiamethoxam (limit of thiamethoxam quantification = 0.3 ng/mL).

In 2018, high or abnormal real values were determined as a whole. It was questionable, as there was no correlation between analytical results and mortality or homing performances results. Some sucrose solutions were re-analysed but same results were obtained. For the expected nominal dose of 1.5 ng per bee, two extreme outliers values of 3.828 and 5.142 ng per bee were determined after analytical analyses (Table 14). These two values were completely out of the dose range and were excluded for data treatment.

For the ring test 2019, it was asked to keep the stock solution prepared in acetone to analyse them in case of problems with the testfeeding solution or in case of abnormal values. In 2019, real doses analysed from sucrose solution were more concordant with what was expected (Table 15).

Table 14: Results of the analytical analyses of the test feeding solutions for each test run and laboratory in 2018

Lab	Run	Nominal dose : 1.5 ng/bee	Nominal dose : 1 ng/bee	Nominal dose : 0.33 ng/bee
1	1	5.142	1.508	0.478
	2	2.008	1.406	0.482
	3	3.828	1.494	0.510
2	1	1.772	1.368	0.442
	2	1.330	0.946	0.294
	3	1.788	1.220	0.406
3	*1	2.026	1.366	0.460
	*2	1.324	0.640	0.252
	*3	1.250	1.062	0.246
4	1	1.640	1.158	0.374
	2	1.962	1.390	0.456
	3	2.216	1.572	0.608
5	*1	2.658	1.938	0.622
	2	2.432	1.790	0.706
	*3	2.734	1.888	0.614
6	1	2.242	1.662	0.570
	2	2.174	1.580	0.560
	3	1.286	0.970	0.338
7	1	1.914	1.418	0.576
	2	1.694	0.854	0.358
	3	1.966	1.310	0.352
8	1	1.824	1.396	0.420
	2	2.042	1.408	0.498
	3	2.096	1.516	0.506

* Invalid test runs because of low homing success of control bees (< 60%)
In red: outliers

Table 15: Results of the analytical analyses of the test feeding solutions for each test run and laboratory in 2019

Lab	Run	Nominal dose : 1.5 ng/bee	Nominal dose : 1 ng/bee	Nominal dose : 0.33 ng/bee
1	**1**	1.274	1.048	0.348
	2	1.530	1.082	0.372
	3	1.368	1.056	0.306
2	**1**	1.870	1.146	0.484
	2	2.138	1.340	0.494
	3	2.012	1.386	0.478
3	**1**	1.988	1.404	0.544
	2	1.778	1.264	0.420
	3	1.778	1.230	0.448
4	**1**	2.004	1.470	0.512
	2	1.978	1.364	0.528
5	**1**	1.724	1.154	0.328
	2	1.866	1.450	0.484
	3	1.668	1.022	0.460
6	***1**	NA**	1.382	0.456
	2	1.904	1.312	0.458
	3	1.864	1.092	0.398
7	***1**	1.990	1.448	0.480
	***2**	1.994	1.644	0.540
	***3**	2.110	1.508	0.600
8	***1**	1.764	1.126	0.488
	***2**	1.714	1.328	0.614
	***3**	1.466	1.278	0.690

***** Invalid test runs because of low homing success of control bees (< 60%)
****** No sample for the analyses

5.5 Homing success per treatment

To assess homing success per treatment, data of the three test runs were pooled for labs where all three test runs fulfilled the validity criteria (when homing performance of control bees were ≥ 60 % in individual test runs). In 2019, two valid test runs where considered for one lab that could only perform two runs and for another one with one invalidated run out of three performed.

In 2018, Homing success didn't significantly differ between groups of control bees and groups of bees exposed to 0.33 ng per bee of thiamethoxam (Chi² tests; $P > 0.05$). But homing performances significantly differ between control bees and bees exposed to the highest doses of 1 or 1.5 ng per bee of thiamethoxam. Bees exposed to these highest doses returned to the hive at a significantly lower

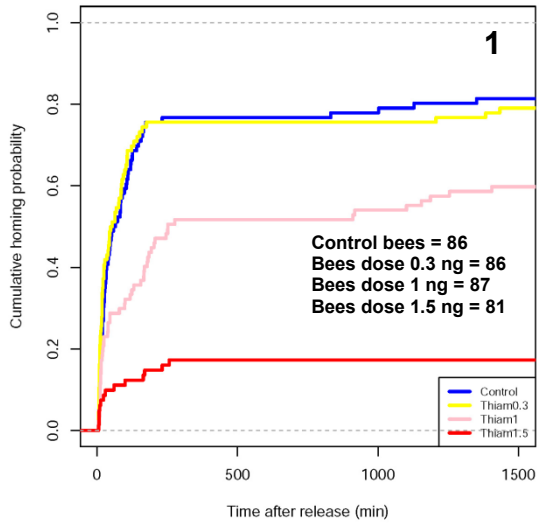
rate compared to control bees or to 0.33-ng exposed bees (Chi² tests; P < 0.05; Table 16, and Figure 11).

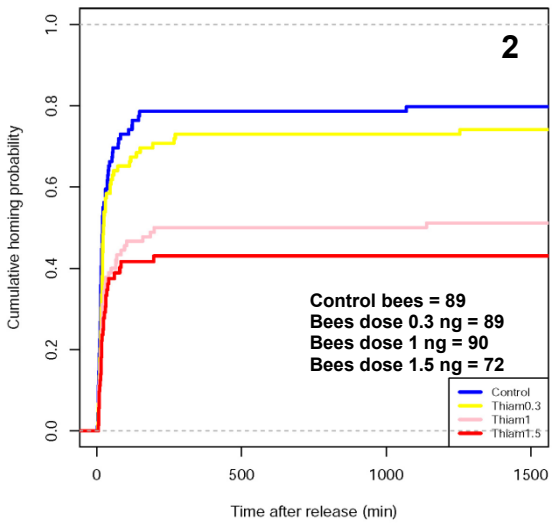
Then, a nominal No Observed Effect Dose (NOED) on the homing success of 0.33 ng per bee for 3 labs and 1 ng per bee for 2 labs could be determined.

Table 16: Homing success results for the ring test 2018 (three valid test runs pooled)

		Control	Thiam. 0.33 ng/bee	Thiam. 1 ng/bee	Thiam. 1.5 ng/bee
Lab 1	Number of foragers released	86	86	87	81
	Homing success probability (24 h after release) [1]	0.814 (a)	0.791 (ab)	0.598 (b)	0.185 (c)
	Chi² Test	Chi² = 87.716, df = 3, P < 2.2e-16			
Lab 2	Number of foragers released	89	89	90	72
	Homing success probability (24 h after release) [1]	0.798 (a)	0.742(a)	0.511(b)	0.431(b)
	Chi² Test	Chi² = 33.219, df = 3, P = 2.895e-07			
Lab 4	Number of foragers released	89	87	78	76
	Homing success probability (24 h after release) [1]	0.708 (a)	0.644 (ab)	0.513 (abc)	0.395 (c)
	Chi² Test	Chi² = 19.415, df = 3, P = 0.0002244			
Lab 6	Number of foragers released	116	108	114	106
	Homing success probability (24 h after release) [1]	0.724 (a)	0.722 (a)	0.351 (b)	0.255 (b)
	Chi² Test	Chi² = 79.931, df = 3, P < 2.2e-16			
Lab 8	Number of foragers released	122	117	125	118
	Homing success probability (24 h after release) [1]	0.910 (a)	0.949 (a)	0.928 (a)	0.746 (b)
	Chi² Test	Chi²= 29.882, df = 3, P = 1.461e-06			

[1]Pairwise comparisons were performed with Chi² test and used Bonferroni P value adjustment method. Same letters indicate no significant differences.





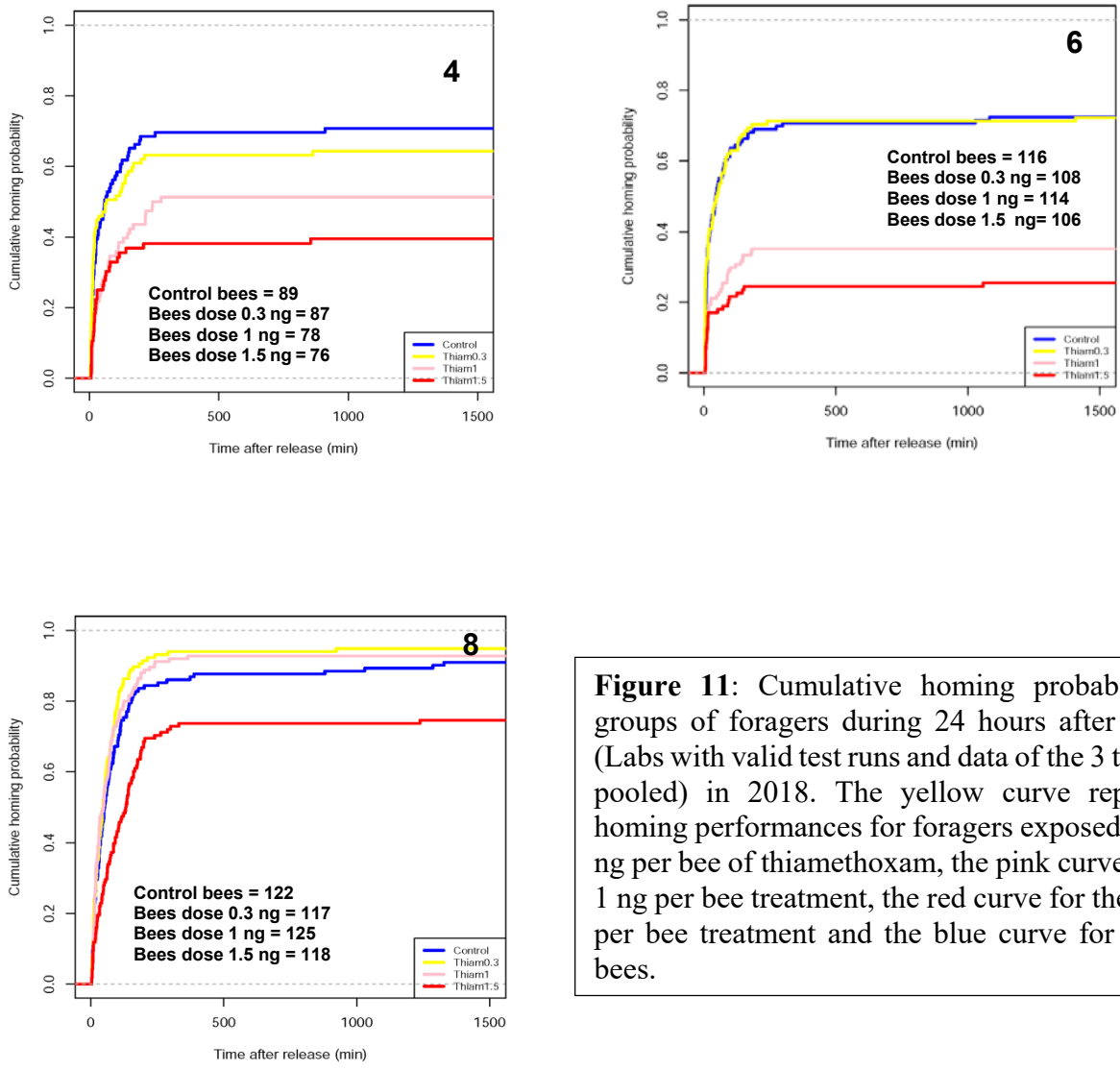


Figure 11: Cumulative homing probability of groups of foragers during 24 hours after release (Labs with valid test runs and data of the 3 test runs pooled) in 2018. The yellow curve represents homing performances for foragers exposed to 0.33 ng per bee of thiamethoxam, the pink curve for the 1 ng per bee treatment, the red curve for the 1.5 ng per bee treatment and the blue curve for control bees.

In 2019, Homing success also didn't significantly differ between groups of control bees and groups of bees exposed to 0.33 ng per bee of thiamethoxam (Chi² tests; $P > 0.05$). Homing performances significantly differ between control bees and bees exposed to the highest doses of 1 or 1.5 ng per bee of thiamethoxam. Bees exposed to these highest doses returned to the hive at a significantly lower rate compared to control bees or to 0.33-ng exposed bees (Chi² or tests; $P < 0.05$; Table 17, and Figure 12).

Then, a nominal No Observed Effect Dose (NOED) on the homing success of 0.33 ng per bee for 4 labs to 1 ng per bee for 2 labs could be determined.

Table 17: Homing success results for the ring test 2019 (two to three valid test runs pooled)

		Control	Thiam. 0.33 ng/bee	Thiam. 1 ng/bee	Thiam. 1.5 ng/bee
Lab 1	Number of foragers released	84	85	80	79
	Homing success probability (24 h after release) [1]	0.810 (a)	0.835 (a)	0.700 (a)	0.278 (b)
	Chi² Test	Chi²= 71.982, df = 3, P = 1.606e-15			
Lab 2	Number of foragers released	89	88	90	80
	Homing success probability (24 h after release) [1]	0.719 (a)	0.636 (ab)	0.467 (bc)	0.325 (c)
	Chi² Test	Chi²= 31.633, df = 3, P = 6.255e-07			
Lab 3	Number of foragers released	85	82	81	82
	Homing success probability (24 h after release) [1]	0.835 (a)	0.854 (a)	0.593 (b)	0.256 (c)
	Chi² Test	Chi² = 83.17, df = 3, P < 2.2e-16			
Lab 4*	Number of foragers released	79	76	75	79
	Homing success probability (24 h after release) [1]	0.772 (a)	0.763 (a)	0.600 (ab)	0.494 (b)
	Chi² Test	Chi² = 18.881, df = 3, P = 0.0002893			
Lab 5	Number of foragers released	114	114	115	116
	Homing success probability (24 h after release) [1]	0.939 (a)	0.904 (a)	0.730 (b)	0.647 (b)
	Chi² Test	Chi² = 42.454, df = 3, P = 3.214e-09			
Lab 6*	Number of foragers released	59	59	60	52
	Homing success probability (24 h after release) [1]	0.729 (a)	0.712 (a)	0.417 (b)	0.192 (b)
	Chi² Test	Chi² = 43.956, df = 3, P = 1.542e-09			

[1]Pairwise comparisons were performed with Chi² test and used Bonferroni P value adjustment method. Same letters indicate no significant differences. * Data from two valid test runs pooled.

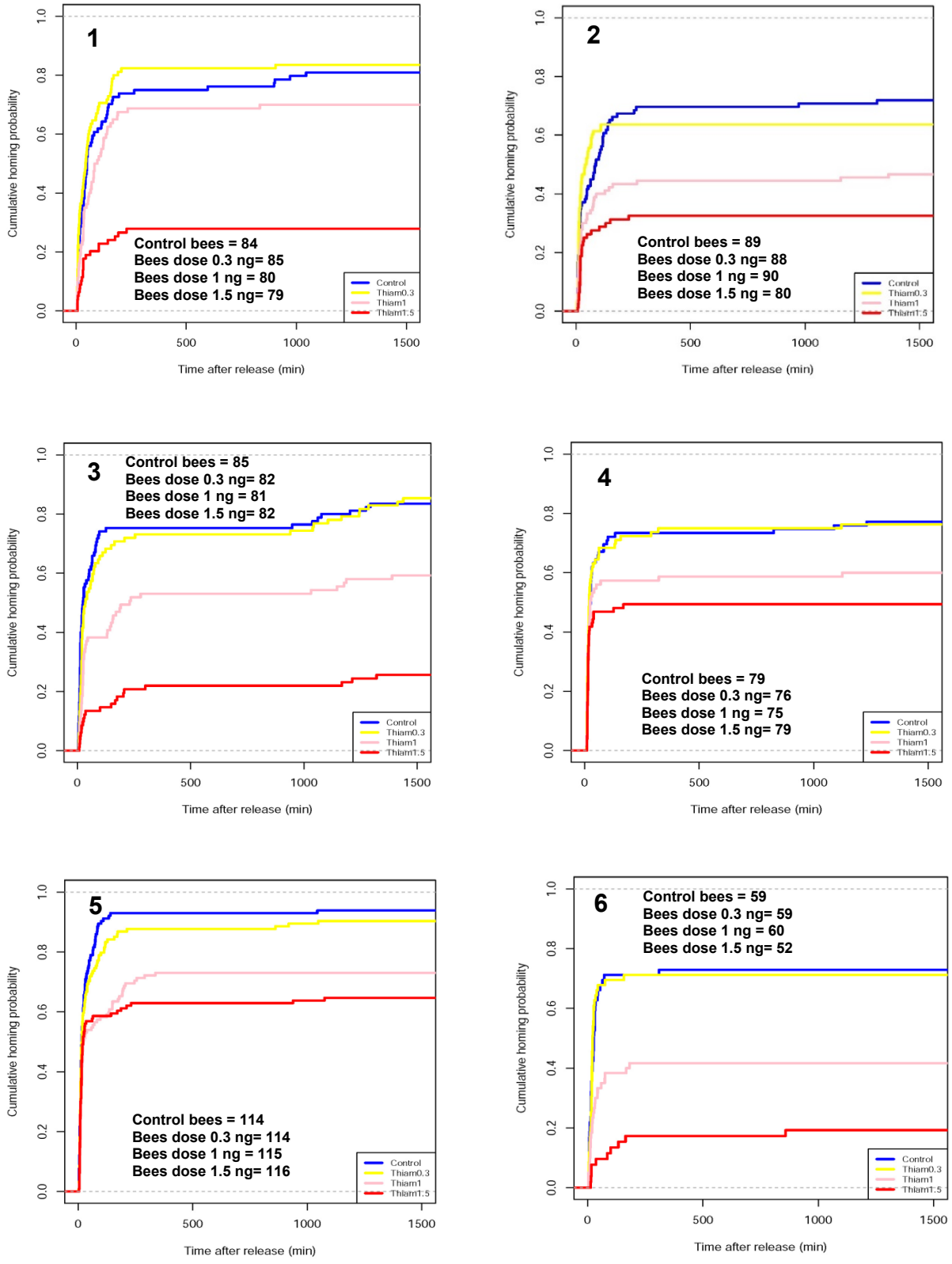


Figure 12: Cumulative homing probability of groups of foragers during 24 hours after release (Labs with valid test runs and data of 2 to 3 test runs pooled) in 2019. The yellow curve represents homing performances for foragers exposed to 0.33 ng per bee of thiamethoxam, the pink curve for the 1 ng per bee treatment, the red curve for the 1.5 ng per bee treatment and the blue curve for control bees.

Details of statistical analyses for homing performance of each laboratory are presented in **Appendix 7**.

Summary of the test endpoint (NOED) determination for the five years of ring test

As previously written, better results concerning validity were obtained in 2017. But feeding the bees before release increased the homing results variability in exposed bees and no NOED could be determined for nearly 29 % of the tests (Table 18). We confirm that the protocol applied in 2019 is the best compromise considering test validity and NOED determination.

Table 18: Percentage of tests for which a NOED could be determined or not from 2 to 3 valid test runs pooled together (homing performances of control bees ≥ 60 %)

	NOED determined (%)	No NOED determined (%)	Invalid Tests (%)
2015 (n=7 tests)	42.9	28.6*	28.6
2016 (n=11 tests)**	45.4	27.3	27.3
2017 (n=7 tests)**	57.1	28.6	14.3
2018 (n=8 tests)	62.5***	0	37.5
2019 (n=8 tests)	75.0****	0	25.0

* Labs that fed the bees after exposure and before release
** Feeding period before release in the protocol 2016 and 2017
*** NOED of 0.33 ng/bee for 60 % of the valid tests (n = 5 tests in 2018)
**** NOED of 0.33 ng/bee for 66.7 % of the valid tests (n= 6 tests in 2019)

5.6 Homing duration per treatment

As a secondary observation, we calculated homing duration per treatment 24 hours after release. Test runs data were pooled for labs where all three test runs fulfilled the validity criteria (when homing performance of control bees were ≥ 60 % in individual test runs). Like for homing performances, two valid test runs where considered for one lab that could only perform two runs and for another one with one invalidated run out of three performed in 2019.

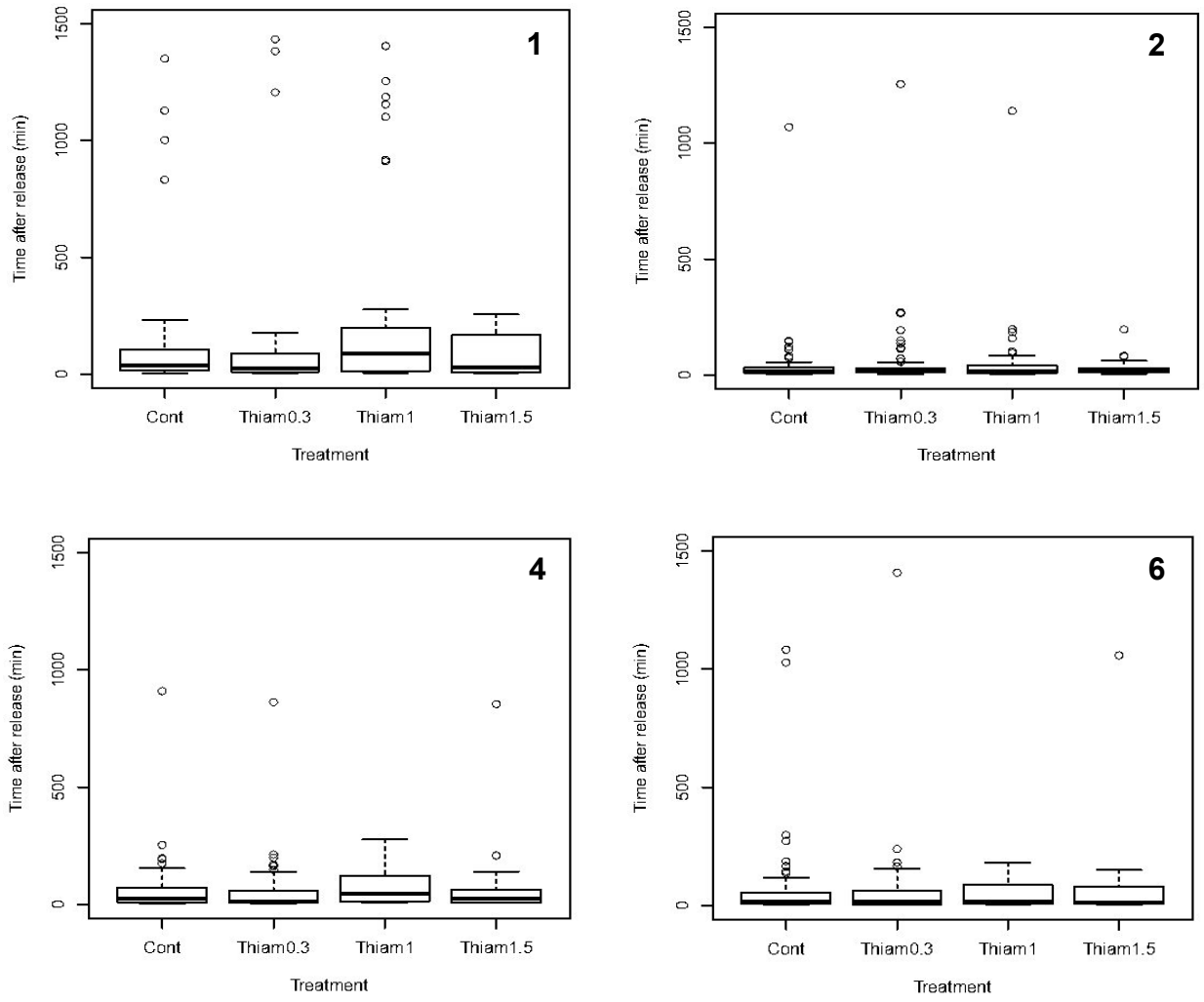
For all the labs in 2018, homing duration didn't significantly differ between groups of control bees and groups of bees exposed to 0.33 ng per bee of thiamethoxam (Kruskal-Wallis tests followed by Mann-Whitney tests; $P > 0.05$; Table 19 and Figure 13). For 3 labs out of 5, homing duration was significantly longer for the bees exposed to the highest doses (1 or 1.5 ng per bee) compared to control

bees or bees exposed to the lowest dose (0.33 ng per bee) (Kruskal-Wallis tests followed by Mann-Whitney tests; $P < 0.05$; Table 19 and Figure 13).

Table 19: Median homing duration for the ring test 2018 (three valid test runs pooled)

		Control	Thiam. 0.33 ng/bee	Thiam. 1 ng/bee	Thiam. 1.5 ng/bee
Lab 1	**Median homing duration in min (24 h after release)[1]**	**36.58 (ab)**	**24.61 (a)**	**88.64 (b)**	**29.57 (ab)**
Lab 2		14.80 (a)	18.62 (a)	16.77 (a)	16.43 (a)
Lab 4		**23.88 (ab)**	**13.82 (a)**	**46.28 (b)**	**23.67 (ab)**
Lab 6		15.59 (a)	15.55 (a)	16.87 (a)	13.72 (a)
Lab 8		**43.62 (ab)**	**42.07 (b)**	**33.19 (b)**	**80.26 (a)**

[1]For homing duration, Kruskal-Wallis tests were performed. When a significant difference was found ($P < 0.05$), pairwise comparisons were performed using Mann-Whitney tests and Bonferroni P value adjustment method. Same letters indicate no significant differences.



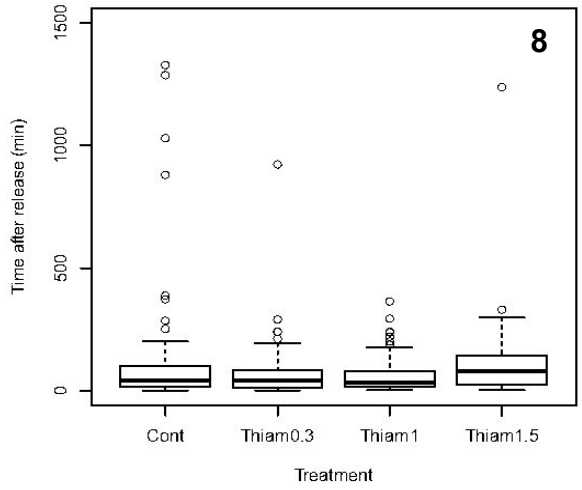


Figure 13: Homing duration of groups of foragers 24 hours after release in 2018 (three valid test runs pooled).

For all the labs in 2019, homing duration didn't significantly differ between groups of control bees and groups of bees exposed to thiamethoxam (Kruskal-Wallis tests followed by Mann-Whitney tests; $P > 0.05$; Table 20 and Figure 14).

Table 20: Median homing duration for the ring test 2019 (two to three test runs pooled)

		Control	Thiam. 0.33 ng/bee	Thiam. 1 ng/bee	Thiam. 1.5 ng/bee
Lab 1	**Median homing duration in min (24 h after release)[1]**	37.48 (a)	31.47 (a)	42.57 (a)	31.90 (a)
Lab 2		23.37 (a)	14.19 (a)	20.33 (a)	19.23 (a)
Lab 3		18.73 (a)	25.45 (a)	26.25 (a)	35.58 (a)
Lab 4		17.60 (a)	16.31 (a)	15.28 (a)	14.27 (a)
Lab 5		13.37 (a)	12.92 (a)	13.96 (a)	12.50 (a)
Lab 6		17.53 (a)	18.35 (a)	19.17 (a)	60.75 (a)

[1]For homing duration, Kruskal-Wallis tests were performed. When a significant difference was found ($P < 0.05$), pairwise comparisons were performed using Mann-Whitney tests and Bonferroni P value adjustment method. Same letters indicate no significant differences.

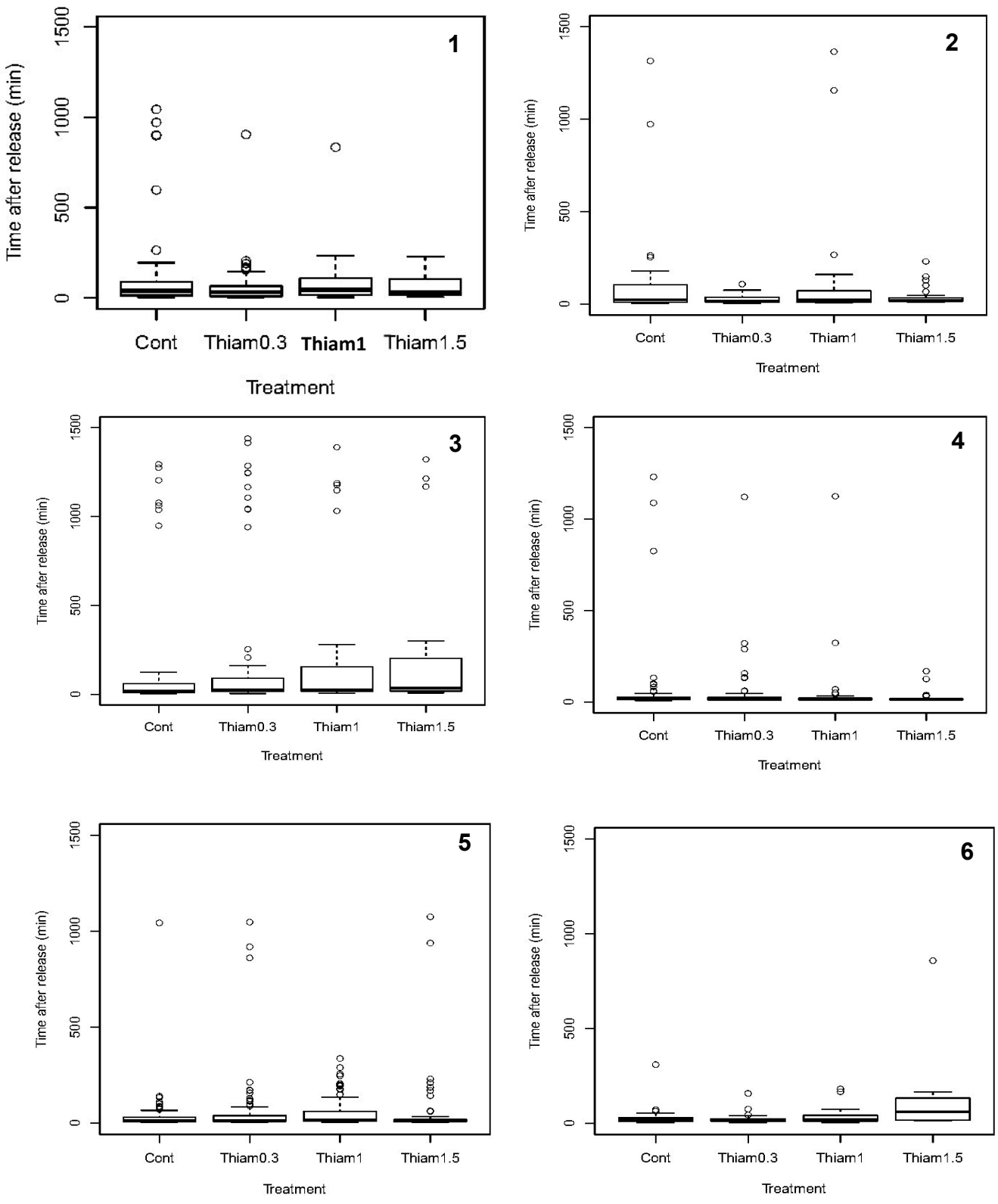


Figure 14: Homing duration of groups of foragers 24 hours after release in 2019 (two to three valid test runs pooled).

Details of statistical analyses for homing duration of each laboratory are presented in **Appendix 7**.

5.7 Variability of homing performances

Homing results, especially in exposed bees, may be modulated by different factors linked to environmental conditions (temperature and landscape, Henry et al. 2014) or health status of the colony, particularly Varroa (Monchanin et al. 2019) as homing performances are measured under field conditions.

Analyses were performed considering valid test runs only (**17 in 2018 and 16 in 2019**) and real doses to which the bees were exposed. The additional explanatory variables were punctual temperature at the release time, number of landmarks that the bees can cross during the travel back to the colony (indicator of landscape complexity), and number of Varroa per 100 bees (see **part 4.11**).

From the ring test data 2018, Generalized Linear Mixed Effects Models (GLMMs) showed no significant effect of environmental factors (temperature and landscape) alone or in interaction with treatment on homing performance of the bees (Table 21). But Varroa had a significant negative effect alone or in interaction with treatment. Results are illustrated in the Figures 15 and 16. Figure 15 is performed from raw data of valid test runs. Figure 16 is the model prediction of the reference item dose-response function of homing failure probability computed for different combinations of Varroa pressure. Results point to an aggravation of the homing failure of exposed bees with an increase of Varroa infestation of the colonies.

Table 21: Summary of the generalized linear mixed effect models (GLMM) performed on valid test runs to assess the effect of thiamethoxam dose, Varroa, temperature and landscape parameters as well as their interactions on honeybee homing success in 2018*.

GLMM Model parameter	Multimodel averaged estimate ± s.e.	Z	P-value
Intercept	**1.482 ± 0.603**	**2.457**	**< 0.05**
Dose	**-1.590 ± 0.610**	**2.605**	**< 0.01**
Landscape	0.905 ± 0.831	1.089	0.276
Varroa	**-0.970 ± 0.486**	**1.997**	**< 0.05**
Temperature	0.400 ± 0.639	0.626	0.531
Dose x Landscape	-1.213 ± 0.761	1.594	0.111
Dose x Temperature	-0.905 ± 0.693	1.305	0.192
Dose x Varroa	**-1.410 ± 0.490**	**2.876**	**< 0.01**

* Data associated with the two extreme real doses values (outliers, see **part 5.4**) were excluded from the analyses
s.e: Standard error
Z: Test statistic to assess if variables have a significant effect on homing performance

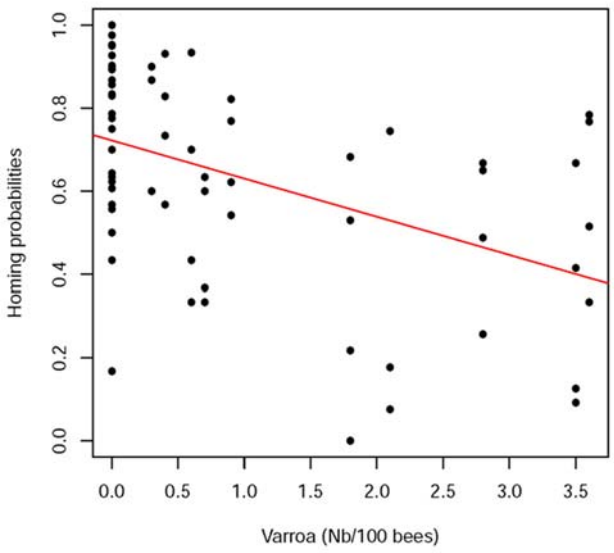


Figure 15: Relationships between homing probabilities of the foragers 24 hours after release and Varroa infestation of the colony (Nb of varroa per 100 bees). A red regression line was added.

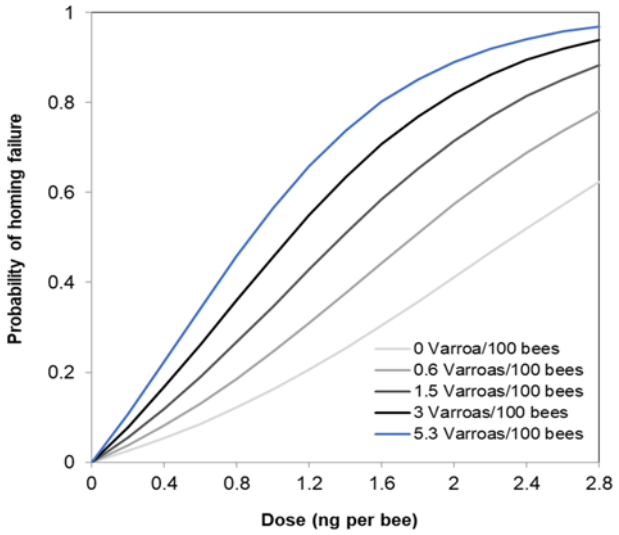


Figure 16: Model prediction of the referent item dose-response function of homing failure probability. The predicted dose-response curves were computed for different combinations of Varroa pressure ranging from no to more than 5 Varroas per 100 bees.

In 2019, GLMMs showed again no significant effect of temperature and landscape alone or in interaction with treatment on homing performance of the bees (Table 22). Varroa had a negative but not significant effect as a whole. However, when considering Varroa in interaction with treatment, a significant and positive effect was found (Table 22 and Figure 17). In 2019, the main majority of colonies tested had low or null Varroa infestation (**Appendix 8**). Only Lab 4 had highest varroa infestation (more than 5 varroas per 100 bees). But homing performances were not affected, especially in exposed bees, compared to the other labs. For the second test run of lab 4, we note high homing performances for bees exposed to the highest doses too (part 5.2, Table 11).

Table 22: Summary of the generalized linear mixed effect models (GLMM) performed on valid test runs to assess the effect of thiamethoxam dose, Varroa, temperature and landscape parameters as well as their interactions on honeybee homing success in 2019.

GLMM Model parameter	Multimodel averaged estimate ± s.e.	Z	P-value
Intercept	1.994 ± 0.395	**5.049**	**< 0.0001**
Dose	**-2.903 ± 0.298**	**9.755**	**< 0.0001**
Landscape	-0.103 ± 0.673	0.154	0.878
Varroa	-0.823 ± 0.714	1.152	0.249
Temperature	0.274 ± 0.950	0.288	0.774
Dose x Landscape	0.122 ± 0.492	0.248	0.804
Dose x Temperature	-0.429 ± 0.784	0.547	0.584
Dose x Varroa	**1.116 ± 0.449**	**2.486**	**< 0.05***

*P value = 0.013
s.e: standard error
Z: _Test statistic to assess if variables have a significant effect on homing performance

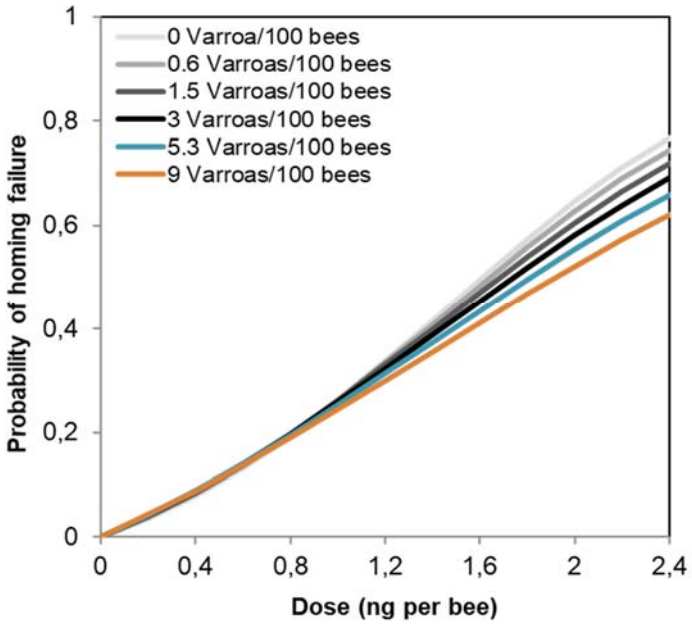


Figure 16: Model prediction of the reference item dose-response function of homing failure probability. The predicted dose-response curves were computed for different combinations of Varroa pressure ranging from no to 9 Varroas per 100 bees.

Varroa is one factor that may modulate homing performances especially in exposed bees. The parasite must be controlled as far as possible. In 2018, accepted valid test runs (n= 17) are not impacted with a varroa infestation at least equal or below to 4 and 5 varroas per 100 bees (Table 23). It also has to be noted that 2 invalid test runs (homing performances in control bees < 60%) performed in late August/beginning of September had a varroa pressure above 6 varroas per 100 bees (6.7 and 8.1 varroas per 100 bees, see annex 8 for 2018). Then, considering that the homing flight test may be performed from April to September (according to weather conditions and availability of blooming crops) and that varroa pressure may evolve during this time with the colony development, an acceptable infestation threshold of the colonies for the test could be **< 5 varroas per 100 bees**.

Table 23: Number of accepted and non-accepted valid test runs when varroa infestation of the colonies were less or equal to 3, 4 or 5 varroas per 100 bees

	≤ 3 varroas/100 bees	**≤ 4 varroas/100 bees**	**≤ 5 varroas/100 bees**
Accepted test runs	15	17	17
Not accepted test runs	2	0	0

This threshold of < **5 varroas per 100 bees** can be compared to informations found in literature. The most cited economic threshold warning for mortality risk of the colony and decreasing honey production ranged from 3 200 to 4 200 varroas per colony (Delaplane et al. 1999). Then, considering an average of 3700 varroas per colony, this is in accordance to a maximum of 4 to 5 varroas per 100 bees according to honeybee population size (http://extension.msstate.edu/sites/default/files/publications/publications/p2826_web.pdf; Table 1). In France, above 5 varroas per 100 bees, honey production from lavender crop can decrease to an average of 6.5 kg less per colony compared to colonies with less than 3 varroas per 100 bees. (Kretzschmar et al. 2016).

Varroa and Landscape data obtained in 2018 and 2019 are proposed in **Appendix 8**. Details of the GLMMs results are presented in **Appendix 9**.

5.8 Critical points with the homing flight method

From the questionnaire 2018, one objective was to try to identify possible remaining problems with the homing flight method, especially for labs that encounter difficulties to perform the test as a whole.

Problems	**Protocol 2018**	**Improvement**
Use of cold blocks directly with collected bees to transport them to the laboratory (2 labs in 2018)	No mention of cold blocks	Impact on the bees' maintenance during lab phase ➔ No use of cold block mentioned in the protocol 2019

Problems	Protocol 2018	Improvement
Collection of the foragers before coloring and release 1) Collection of foragers only going out the hive (1 lab in 2018 and 2019) 2) Collection of foragers only carrying nectar when entering the hive (1 lab in 2018)	All types of foragers carrying pellets of pollen or not (nectar or pollen)	Need to precise the type of foragers collected: 1) Capture of foragers entering the hive only because foraging trip is performed and bees are so expected to come back quicker to the hive after coloration (pink powder) and first release 2) Capture as far as possible of pollen collectors with expected low stomach content i) for better consumption and homogenization among bees via trophallaxis during exposure phase; ii) to prevent possible dilution that may occur when only nectar foragers with expected higher stomach content are collected (R&D results of 2 labs in 2018). ➔ Focus on pollen collectors was mentioned in the protocol 2019
Difficulties to label all the bees with RFID tags in 1h30 (1 lab in 2018 and 2019) More sucrose solution may be needed during exposure phase when the majority or all foragers are pollen collectors (Results of 1 lab in 2019)	Decrease of the labelling phase from 2h00 to 1h30 for the maintenance of the bees Not discussed for the protocol 2018; discussed after the ring test 2019	Labelling the bees in two hours may be risky (e.g; weak bees before the exposure phase) Training with the method, especially for the tricky phase of labelling is important For the draft TG, a list of conditions to increase the performance of successful homing test will be proposed (e.g. training with the method, especially labelling; exposure with 20 to 40* µl per bee of sucrose solution according to the bees'needs) *especially when only pollen foragers are used and according to the requirement of the bees.
Homing results may be different according to the mode of exposure: individual vs collective (10 bees feeding scheme)	From 2015, bees are exposed collectively as no differences were found between individual *vs* collective exposure (see Results of the First ring test 2015) but other authors showed more variability in homing performances with the collective exposure (Jeker & Grossar, 2020)	Collective exposure (10 bees feeding scheme) is kept for the homing flight test, for following reasons: - Differences between individual and collective exposure are not always observed, - Individual exposure is more time-consuming (caging, check of syrup consumption, release in field), -More people would be asked for the bees ' manipulation (e.g. at least one to two persons more for the labelling phase).

CONCLUSION

As a whole, the ring test results showed that the homing flight test matches different points for validation:
- **Feasibility**: a great majority of the labs could conduct the test with success (73 % of valid tests out of 41 tests performed over the 5 ring test years[2])
- **Sensitivity**: to detect of effects of sublethal doses of thiamethoxam on homing performances of foragers compared to control bees (77 % on 30 valid tests over the 5 ring test years)
- **Results reproducibility**: a majority of labs established the test endpoint (NOED in ng per bee) (77 % on 30 tests performed with success over the 5 ring test years; all the labs with successful tests determined a NOED in 2018 and 2019)

Validity criteria:
For each test run, mortality of the bees after exposure and before release met the validity criterion of ≤ 15% as a whole for control but also for exposed bees. The second validity criterion, the minimum and acceptable homing performances in control bees, showed to vary between 60 and 70 % according to the ring test results. Best homing success in control bees were obtained in 2017 but bees were fed *ad libitum* and results variability increased in exposed bees because of the dilution of the remaining stomach content. The last ring test year (2019) is a compromise for the NOED determination and for the homing performances of control bees. In 2019, nearly 70 % of the valid test runs met a minimum and acceptable homing success in control bees of ≥ 60 %. But when increasing the minimum and acceptable control rate to ≥ 70%, similar results were obtained with 65 % of valid test runs.

Comparison of the ring test results with "natural" foragers' loss under field condition is important to bring supplementary data (F. Requier, com. pers.). R&D study presented in this report showed that foragers' survival mainly vary between 70 and 100 %. Then, this study would support a maximum daily foragers' loss of 30 %. This is comparable to the minimum and acceptable level of 30 to 40 % of homing failure for the control bees in the homing test.

Factors of variability:
Environmental factors such as temperature and landscape, (number of landmarks) and Varroa were considered alone or in interaction with treatment to assess their possible effect in homing results variabilities, especially for exposed bees. No significant effect of temperature or landscape were found from the ring test data 2018 and 2019. But Varroa showed significant negative effects alone or in interaction with treatment in 2018. Varroa may modulate homing performances especially in exposed bees. As far as possible, colonies with low Varroa infestation are recommended to perform the test (**< 5 varroas per 100 bees** considering results 2018 and literature recommendations).

[2] We considered tests with 2 to 3 valid runs (homing success of control bees ≥ 60 %)

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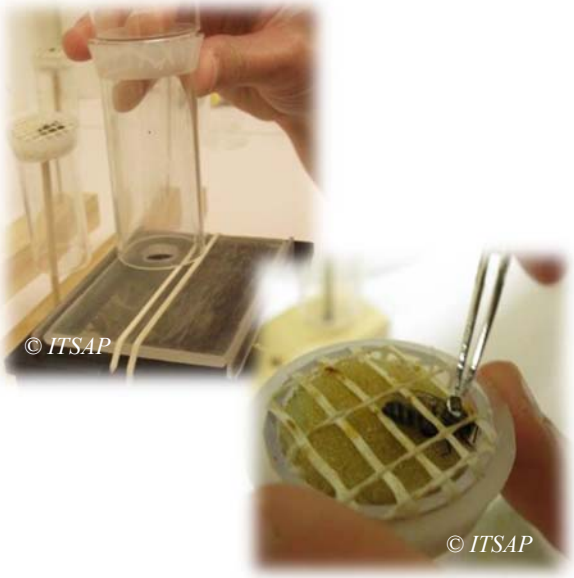
APPENDIX 1

Capture of the pink powdered bees at the hive entrance after release



Transfer to a holding cage and labelling with a RFID tag

Exposure phase per small group of 10 bees

Example of a RFID system for the homing flight ring test

APPENDIX 2

Protocol to control performance of the RFID system

- 6 « test » tags glued onto small plastic or wooden sticks ➔ UIDs of the tags first recorded
- Each tag is passed five times through each of the four readers ➔ 20 readings per tag expected and a total of 120 readings expected for the 6 test tags
- Tested tag must be read at least one time each time it passes through a reader
- Reading rates (%) is calculated as recorded data on expected data (120 readings)

The acceptance criteria for the performance of the RFIS system was that **at least 95% of the crossing of the tags should be recorded**.

Reading rate control of the RFID system 2018

Laboratory	Date	Total number of reading	Reading rate (%)
Lab 1	28/05/2018	120	100
Lab 2	09/07/2018	120	98.33
Lab 3	25/07/2018	120	100
Lab 4	29/06/2018	120	99.17
Lab 5	02/07/2018	120	99.17
Lab 6	13/08/2018	120	100
Lab 7	24/08/2018	120	97.50
Lab 8	29/05/2018	120	100

Reading rate control of the RFID system 2019

Laboratory	Date	Total number of reading	Reading rate (%)
Lab 1	13/05/2019	120	99.17
Lab 2	18/04/2019	120	98.33
Lab 3	03/06/2019	120	100
Lab 4	08/07/2019	120	100
Lab 5	24/04/2019	200	98.00
Lab 6	26/06/2019	120	98.33
Lab 7	20/06/2019	120	98.33
Lab 8	12/07/2019	6	100

APPENDIX 3

2018 and 2019

CLUZEAU INFO LABO
33-35 Rue Jean-Louis Faure, BP 88
33220 SAINTE-FOY-LA-GRANDE
☎ : 05 57 46 00 44

Dr. Ehrenstorfer
Reference Materials for Residue Analysis

Certificate of Analysis

ISO Guide 34 Reference Material

Product Identification

Articel Code:	DRE-C17453000
Artikel Name:	Thiamethoxam
Formula:	C8H10ClN5O3S
Mol. Weight:	291.71
CAS No.:	153719-23-4

Lot Number:	G133046
Expiry Date:	16.01.2021
Storage Temperature:	20°C ± 4°C

Storage and handling: The RM should be stored in the original sealed bottle at the temperatur given above. After use the bottle should be tightly closed and protected from moisture and light. The expiry date is valid for original closed bottles under recommended storage conditions only.

Purity	99.69%
Expanded Uncertainty U=	0.30%

The uncertainty of this standard is calculated in accordance with the ISO Guide 34 and EURACHEM/CITAC Guide - Quantifying Uncertainty in Analytical Measurement, Second Edition. The Expanded uncertainty is U = u(RM) x k, where k i the coverage factor at the 95% confidence Level (k=2). The expanded uncertainty U is bades on the combination of the uncertainties associated with each individual operation involved in the analysis of the product. $U(RM) = \sqrt{u(char)^2 + u(bb)^2 + u(lts)^2 + u(sts)^2}$; u(char) is the uncertainty of purity determination; u(bb) uncertainty of homogeneity test; u(lts) is uncertainty of stability test long-term; u(sts) is uncertainty of stability test short-term. u(lts) and u(sts) are not included in the calculation as the stability statement is based on real evidence opposed to simulation.
Minimum sample: 1 mg is recommended as the minimal sample amount. If less material is used, it is recommended to increase the certified uncertainty by a factor of two for half sample and a factor of four for a quarter of sample
Intended use: Use this RM as calibrant for chromatography or any other analytical technique.

Analytical Data

Traceability of chromatography: To the International System of Unity (SI).

Intrument:	HPLC/DAD		Method Details
Detection:	DAD		Acetonitrile:Water 4:1
Column:	ReproSil 100 C18 5 µm 250 x 3 mm		
Inj.-Vol.:	10	µl	
Flow:	1	ml/min	
Ret.Time:	1.15	min	

Comment
Traceability: The balances used are calibrated with weights traceable to the national standards (DKD).
Calibrated Class A glassware is used for volumetric measurements.
Certificate Revision 1
Water Content: 0.26% (g/g) by Karl-Fischer-Titration (U(exp) = 0.22% (g/g)).
Identity: EA, NMR, RT, IR, UV

Certified on: 18.05.2017
Certified by: N. Müller

Authorized copy
from the original
19. MAI 2017
Sign.:

The Laboratory LGC Labor GmbH is accreditated by DAkkS as indicated by the Accreditations Number D-RM-19883-01 & D-PL-19883-01 has shown competence based on ISO Guide 34:2009 with relevant parts of DIN EN ISO/IEC 17025:2005 for production of certified reference materials in form of organic pure substances and in form of single and multi-component solutions of organic pure substances.

LGC Labor GmbH - Bgm.Schlosser-Straße 6A - 86199 Augsburg - Germany
Phone +49 821 906080 - Fax +49 821 9060888 - augsburg.inquiry@lgcgroup.com
The warranty for this product is limited to the purchasing price of this product

Data File L:\GERÄTE BACKUP\DAD3\2017\2017KW02\120117-2 2017-01-12 13-08-35\17453000-1.D

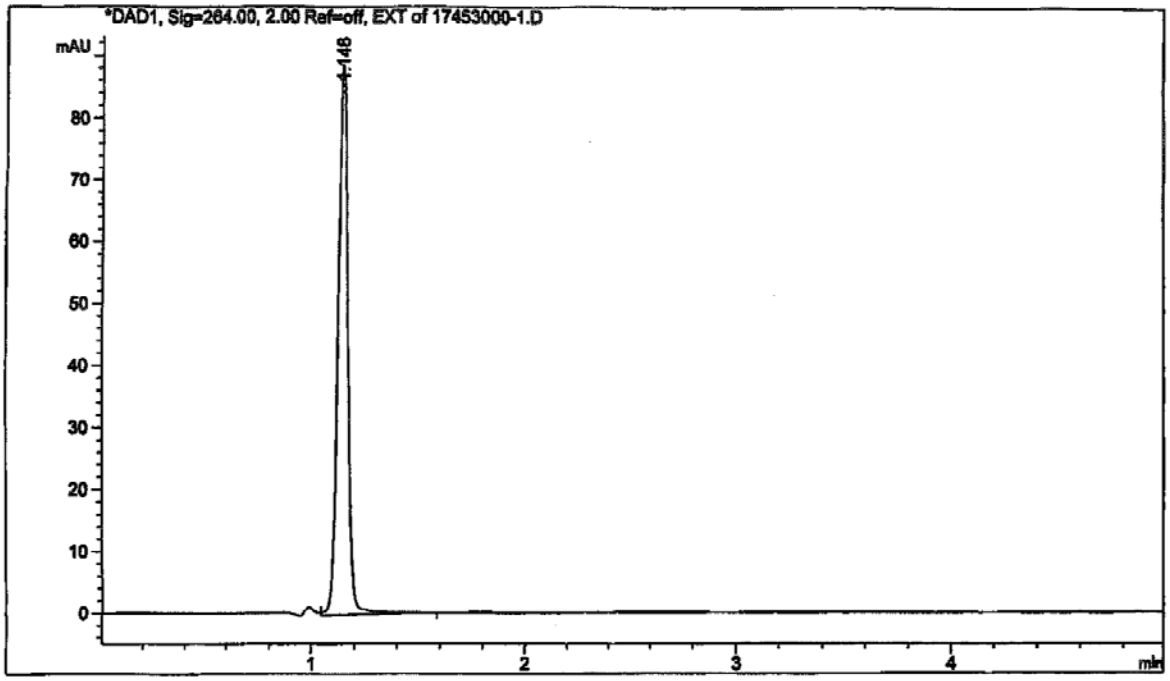
13.01.17 HQ

Acq. Operator : DAD3_Admin
Acq. Instrument : DAD3
Injection Date : 12.01.2017 22:00:40

Seq. Line : 36
Location : 82
Inj : 1
Inj Volume : 10.000 µl

Acq. Method : C:\Chem32\1\DATA\2017KW02\120117-2 2017-01-12 13-08-35\41K.M
Last changed : 08.11.2016 07:28:53 by DAD3_Admin
Analysis Method : L:\GERÄTE BACKUP\DAD4\METHODS\41K.M
Last changed : 29.10.2015 10:40:19 by SYSTEM
Method Info : Acetonitrile : Water 4:1

Sample Info : Thiamethoxam



Area Percent Report

Sorted By : Retention Time
Multiplier : 1.0000
Dilution : 1.0000
Use Multiplier & Dilution Factor with ISTDs

Signal 1: DAD1, Sig=264.00, 2.00 Ref=off, EXT
Signal has been modified after loading from rawdata file!

Peak #	RetTime [min]	Sig	Type	Area [mAU*s]	Height [mAU]	Area %
1	1.146	1	VB	280.75775	89.04220	100.0000
Totals :				280.75775	89.04220	

*** End of Report ***

APPENDIX 4

Preparation of the item, test solutions and test feeding solutions

- ⇨ 20 µl of sucrose solution (30 % w/v) per bee containing 0.1% of acetone is considered
- ⇨ Test item doses: 0.33, 1 and 1.5 ng per bee

1- Preparation of the stock solution (S)

***1.5 ng test item in 0.02 µl acetone => 75 ng/µl or 75 µg/ml*

Preparation of a one hundred time more concentrated « S »:

75 x 100 = 7 500 µg/ml or 7.5 mg/ml

To prepare « S » => 15 mg of thiamethoxam is weighed and 2 ml of acetone is added

2- Preparation of a 1.5 ng per bee test solution (S1)

Dilution 1/100: solution « S1 » at 75 µg/ml

10 ml as a final acetone volume is considered.

Preparation:

Ci xVi = Cf xVf => 7500 µg/ml x Vi = 75 µg/ml x 10

Vi = 0.1 ml => 100 µl of S is sampled and 9.9 ml of acetone is added

3- Preparation of a 1 ng per bee test solution (S2)

Dilution 2/3: solution S2 at 50 µg/ml; 3 ml as a final acetone volume is considered

Preparation :

Ci xVi = Cf xVf => 75 µg/ml x Vi = 50 µg/ml x 3

Vi = 2 ml => 2 ml of S1 is sampled and 1 ml of acetone is added

4- Preparation of a 0.33 ng per bee test solution (S3)

Dilution 1/3: solution S3 at 16.667 µg/ml; 3 ml as a final acetone volume is considered

Preparation : 1 ml of S2 is sampled and 2 ml of acetone is added

5- Test feeding solutions

General preparation: 15 g of sugar in 50 ml of demineralized water (30 % w/v)

Four samples of 10 ml of this sucrose solution are prepared for the 3 tested and control treatments.

Test feeding solutions are prepared in 10 ml of sucrose solution:

Treatment	Test solution sample in µl	Sucrose solution (30% w/v) in ml
Control (acetone)	10 µl acetone	10
Thiamethoxam 1 ng	10 µl S1	10
Thiamethoxam 0.33 ng	10 µl S2	10
Thiamethoxam 0.11 ng	10 µl S3	10

APPENDIX 5

Percentage of pollen foragers captured at the hive entrance for the homing flight ring test 2019

Lab	Run	Pollen foragers (%)
1	1	60
	2	45
	3	40
2	1	90
	2	80
	3	50
3	1	100
	2	100
	3	100
4	1	70
	2	
5	1	18.5
	2	34
	3	28
6	1	90
	2	90
	3	70
7	1	Capture of bees going out the hive
	2	Capture of bees going out the hive
	3	Capture of bees going out the hive
8	1	Not determined Capture of all type of foragers entering the hive
	2	Not determined Capture of all type of foragers entering the hive
	3	Not determined Capture of all type of foragers entering the hive

APPENDIX 6

A) Punctual weather conditions (temperature, hygrometry, cloud layer and wind strength) at the time of the bees release during the homing flight ring test 2018

Lab	Run	Mean temperature (°C)	Mean hygrometry (%)	Cloud layer	Wind strength
1	1	21.5	40	Low	Low
	2	22.4	45	Average	Null
	3	27.2	42	Low	Low
2	1	32.2	37.3	Null	Average
	2	33.5	35.1	High	Null
	3	23.2	46.5	High	Low
3	1	35.6	23.7	Null	Null
	2	31.7	33.5	Null	Null
	3	27.2	30.6	Null	Null
4	1	35.3	25	Null	Null
	2	34.6	10	Null	Null
	3	39.2	21	Null	Null
5	1	29	53	Low	Low
	2	33	42	Low	Low
	3	33	39	Low	Low
6	1	22.1	62	High	Low
	2	27.2	51	Average	Null
	3	28	22	Null	Null
7	1	26.2	41	Low	Low
	2	-	-	Low	Low
	3	31	-	Low	Low
8	1	30.4	48.6	Low	Low
	2	25.5	46.4	Low	Low
	3	28.5	43.6	Low	Low

B) Punctual weather conditions (temperature, hygrometry, cloud layer and wind strength) at the time of the bees release during the homing flight ring test 2019

Lab	Run	Mean temperature (°C)	Mean hygrometry (%)	Cloud layer	Wind strength
1	1	31	19	Null	Null
	2	32.8	15	Low	Low
	3	38.5	16	Null	Null
2	1	26.4	30.2	Low	Null
	2	30.9	45.6	Low	Low
	3	30.0	24.9	Average	Low
3	1	26	42	Low	Low
	2	24	67	Average	Average
	3	26	40	Low	Low
4	1	24.2	49	Null	Null
	2	29.4	42.1	Low	Null
5	1	27.7	43	Low	Null
	2	24.4	43	Average	Low
	3	26.7	50	High	Null
6	1	23	72.5	Low	Low
	2	25	55	Average	Average
	3	26	50	Average	Low
7	1	34	40	Null	Null
	2	34	51	Null	Null
	3	34	33	Null	Null
8	1	28.9	27	Low	Null
	2	27.3	43	Average	Low
	3	27	52	Low	Null

APPENDIX 7

Detail of the statistical analysis performed on homing success and homing duration 24 hours after the release of the bees

2018 – HOMING SUCCESS

Lab 1

```
> tab_cont<-table(ab1$Treat,ab1$rfid_release_min)
> prop.test(tab_cont, alternative='two.sided',conf.level=.95,correct=FALSE)

4-sample test for equality of proportions without continuity correction
data:  tab_cont

X-squared = 87.716, df = 3, p-value < 2.2e-16
alternative hypothesis: two.sided
sample estimates:
   prop 1    prop 2    prop 3    prop 4
0.1860465 0.2093023 0.4022989 0.8148148
```

***Multiple comparisons after Chi² (without P value adjustment)**

```
> pairwise.prop.test(tab_cont, p.adj = "none")
Pairwise comparisons using Pairwise comparison of proportions
data:  tab_cont
```

	Cont	Thiam0.3	Thiam1
Thiam0.3	0.8482	-	-
Thiam1	**0.0032**	**0.0096**	-
Thiam1.5	**1.6e-15**	**1.8e-14**	**1.2e-07**

***P value adjustment method : Bonferroni**

```
> pairwise.prop.test(tab_cont, p.adj = "bonferroni")
 Pairwise comparisons using Pairwise comparison of proportions
data:  tab_cont
```

	Cont	Thiam0.3	Thiam1
Thiam0.3	1.000	-	-
Thiam1	**0.019**	0.058	-
Thiam1.5	**9.7e-15**	**1.1e-13**	**7.0e-07**

Lab 2

```
> tab_cont<-table(ab1$Treat,ab1$rfid_release_min)
> prop.test(tab_cont, alternative='two.sided',conf.level=.95,correct=FALSE)

4-sample test for equality of proportions without continuity
       correction
data:  tab_cont
```

X-squared = 33.219, df = 3, p-value = 2.895e-07

```
alternative hypothesis: two.sided
sample estimates:
   prop 1    prop 2    prop 3    prop 4
0.2022472 0.2584270 0.4888889 0.5694444
```

***Multiple comparisons after Chi² (without P value adjustment)**

```
> pairwise.prop.test(tab_cont, p.adj = "none")
Pairwise comparisons using Pairwise comparison of proportions
data:  tab_cont
```

	Cont	Thiam0.3	Thiam1
Thiam0.3	0.47643	-	-
Thiam1	**0.00011**	**0.00244**	-
Thiam1.5	**3.4e-06**	**0.00012**	0.38875

***P value adjustment method : Bonferroni**

```
> pairwise.prop.test(tab_cont, p.adj = "bonferroni")
  Pairwise comparisons using Pairwise comparison of proportions
data:  tab_cont
```

	Cont	Thiam0.3	Thiam1
Thiam0.3	1.00000	-	-
Thiam1	**0.00065**	**0.01462**	-
Thiam1.5	**2.1e-05**	**0.00072**	1.00000

Lab 4

```
> tab_cont<-table(ab1$Treat,ab1$rfid_release_min)
> prop.test(tab_cont, alternative='two.sided',conf.level=.95,correct=FALSE)

4-sample test for equality of proportions without continuity
       correction
data:  tab_cont
```

X-squared = 19.415, df = 3, p-value = 0.0002244

```
alternative hypothesis: two.sided
sample estimates:
   prop 1    prop 2    prop 3    prop 4
0.2921348 0.3563218 0.4871795 0.6052632
```

***Multiple comparisons after Chi² test (without P value adjustment)**

> pairwise.prop.test(tab_cont, p.adj = "none")

Pairwise comparisons using Pairwise comparison of proportions

data: tab_cont

	Cont	Thiam0.3	Thiam1
Thiam0.3	0.4540	-	-
Thiam1	**0.0152**	0.1228	-
Thiam1.5	**0.0001**	**0.0025**	0.1904

***P value adjustment method: Bonferroni**

> pairwise.prop.test(tab_cont, p.adj = "bonferroni")

Pairwise comparisons using Pairwise comparison of proportions

data: tab_cont

	Cont	Thiam0.3	Thiam1
Thiam0.3	1.00000	-	-
Thiam1	0.09133	0.73660	-
Thiam1.5	**0.00061**	**0.01524**	1.00000

Lab 6

```
> tab_cont<-table(ab1$Treat,ab1$rfid_release_min)
```

> prop.test(tab_cont, alternative='two.sided',conf.level=.95,correct=FALSE)

4-sample test for equality of proportions without continuity correction

data: tab_cont

X-squared = 79.931, df = 3, p-value < 2.2e-16

alternative hypothesis: two.sided

sample estimates:

prop 1	prop 2	prop 3	prop 4
0.2758621	0.2777778	0.6491228	0.7452830

***Multiple comparisons after Chi² test (without P value adjustment)**

> pairwise.prop.test(tab_cont, p.adj = "none")

Pairwise comparisons using Pairwise comparison of proportions

data: tab_cont

	Cont	Thiam0.3	Thiam1
Thiam0.3	1.00	-	-
Thiam1	**2.9e-08**	**6.4e-08**	-
Thiam1.5	**7.2e-12**	**2.0e-11**	0.16

***P value adjustment method: Bonferroni**

```
> pairwise.prop.test(tab_cont, p.adj = "bonferroni")
  Pairwise comparisons using Pairwise comparison of proportions
data:  tab_cont
```

	Cont	Thiam0.3	Thiam1
Thiam0.3	1.00	-	-
Thiam1	**1.8e-07**	**3.8e-07**	-
Thiam1.5	**4.3e-11**	**1.2e-10**	0.97

Lab 8

```
> tab_cont<-table(ab1$Treat,ab1$rfid_release_min)
> prop.test(tab_cont, alternative='two.sided',conf.level=.95,correct=FALSE)
4-sample test for equality of proportions without continuity
      correction
data:  tab_cont
```

X-squared = 29.882, df = 3, p-value = 1.461e-06

```
alternative hypothesis: two.sided
sample estimates:
   prop 1    prop 2    prop 3    prop 4
0.09016393 0.05128205 0.07200000 0.25423729
```

***Multiple comparisons after Chi² test (without P value adjustment)**

```
> pairwise.prop.test(tab_cont, p.adj = "none")
Pairwise comparisons using Pairwise comparison of proportions
data:  tab_cont
```

	Cont	Thiam0.3	Thiam1
Thiam0.3	0.35898	-	-
Thiam1	0.77187	0.68826	-
Thiam1.5	**0.00135**	**3.5e-05**	**0.00022**

***P value adjustment method: Bonferroni**

```
> pairwise.prop.test(tab_cont, p.adj = "bonferroni")
  Pairwise comparisons using Pairwise comparison of proportions
data:  tab_cont
```

	Cont	Thiam0.3	Thiam1
Thiam0.3	1.00000	-	-
Thiam1	1.00000	1.00000	-
Thiam1.5	**0.00811**	**0.00021**	**0.00133**

2019 – HOMING SUCCESS

Lab 1

```
> tab_cont<-table(ab1$Treat,ab1$rfid_release_min)
> prop.test(tab_cont, alternative='two.sided',conf.level=.95,correct=FALSE)
4-sample test for equality of proportions without continuity correction
data:  tab_cont

X-squared = 71.982, df = 3, p-value = 1.606e-15
alternative hypothesis: two.sided
sample estimates:
   prop 1    prop 2    prop 3    prop 4
0.1904762 0.1647059 0.3000000 0.7215190
```

***Multiple comparisons after Chi² test (without p value adjustment)**

```
> pairwise.prop.test(tab_cont, p.adj = "none")
Pairwise comparisons using Pairwise comparison of proportions
data:  tab_cont
```

	Con t	Thiam0.3	Thiam1
Thiam0.3	0.81	-	-
Thiam1	0.15	0.06	-
Thiam1.5	**2.8e-11**	**2.0e-12**	**2.5e-07**

***P value adjustment method : Bonferroni**

```
> pairwise.prop.test(tab_cont, p.adj = "bonferroni")
Pairwise comparisons using Pairwise comparison of proportions
data:  tab_cont
```

	Cont	Thiam0.3	Thiam1
Thiam0.3	1.00	-	-
Thiam1	0.88	0.36	-
Thiam1.5	**1.7e-10**	**1.2e-11**	**1.5e-06**

Lab 2

```
> tab_cont<-table(ab1$Treat,ab1$rfid_release_min)
> prop.test(tab_cont, alternative='two.sided',conf.level=.95,correct=FALSE)
4-sample test for equality of proportions without continuity correction
data:  tab_cont

X-squared = 31.633, df = 3, p-value = 6.255e-07
alternative hypothesis: two.sided
sample estimates:
   prop 1    prop 2    prop 3    prop 4
0.2808989 0.3636364 0.5333333 0.6750000
```

***Multiple comparisons after Chi² test (without p value adjustment)**

```
> pairwise.prop.test(tab_cont, p.adj = "none")
Pairwise comparisons using Pairwise comparison of proportions
data:  tab_cont
```

	Cont	Thiam0.3	Thiam1
Thiam0.3	0.30915	-	-
Thiam1	**0.00102**	**0.03360**	-
Thiam1.5	**6.6e-07**	**0.00011**	0.08451

***P value adjustment method: Bonferroni**

```
> pairwise.prop.test(tab_cont, p.adj = "bonferroni")
Pairwise comparisons using Pairwise comparison of proportions
data:  tab_cont
```

	Cont	Thiam0.3	Thiam1
Thiam0.3	1.00000	-	-
Thiam1	**0.00614**	0.20158	-
Thiam1.5	**4e-06**	**0.00063**	0.50705

Lab 3

```
> tab_cont<-table(ab1$Treat,ab1$rfid_release_min)
> prop.test(tab_cont, alternative='two.sided',conf.level=.95,correct=FALSE)
4-sample test for equality of proportions without continuity correction
data:  tab_cont
```

X-squared = 83.179, df = 3, p-value < 2.2e-16

```
alternative hypothesis: two.sided
sample estimates:
   prop 1    prop 2    prop 3    prop 4
0.1647059 0.1463415 0.4074074 0.7439024
```

***Multiple comparisons after Chi² test (without p value adjustment)**

```
> pairwise.prop.test(tab_cont, p.adj = "none")
Pairwise comparisons using Pairwise comparison of proportions
data:  tab_cont
```

	Cont	Thiam0.3	Thiam1
Thiam0.3	0.90942	-	-
Thiam1	**0.00098**	**0.00038**	-
Thiam1.5	**1.7e-13**	**4.6e-14**	**2.8e-05**

***P value adjustment method: Bonferroni**

```
> pairwise.prop.test(tab_cont, p.adj = "bonferroni")
Pairwise comparisons using Pairwise comparison of proportions
data:  tab_cont
```

	Cont	Thiam0.3	Thiam1
Thiam0.3	1.00000	-	-
Thiam1	**0.00586**	**0.00229**	-
Thiam1.5	**1.0e-12**	**2.8e-13**	**0.00017**

Lab 4

```
> tab_cont<-table(ab1$Treat,ab1$rfid_release_min)
> prop.test(tab_cont, alternative='two.sided',conf.level=.95,correct=FALSE)
4-sample test for equality of proportions without continuity correction
data:  tab_cont
```

X-squared = 18.881, df = 3, p-value = 0.0002893

```
alternative hypothesis: two.sided
sample estimates:
   prop 1    prop 2    prop 3    prop 4
0.2278481 0.2368421 0.4000000 0.5063291
```

***Multiple comparisons after Chi² test (without p value adjustment)**

```
> pairwise.prop.test(tab_cont, p.adj = "none")
Pairwise comparisons using Pairwise comparison of proportions
data:  tab_cont
```

	Cont	Thiam0.3	Thiam1
Thiam0.3	1.00000	-	-
Thiam1	**0.03306**	**0.04793**	-
Thiam1.5	**0.00053**	**0.00097**	0.24497

***P value adjustment method: Bonferroni**

```
> pairwise.prop.test(tab_cont, p.adj = "bonferroni")
Pairwise comparisons using Pairwise comparison of proportions
data:  tab_cont
```

	Cont	Thiam0.3	Thiam1
Thiam0.3	1.0000	-	-
Thiam1	0.1984	0.2876	-
Thiam1.5	**0.0032**	**0.0058**	1.0000

Lab 5

```
> tab_cont<-table(ab1$Treat,ab1$rfid_release_min)
> prop.test(tab_cont, alternative='two.sided',conf.level=.95,correct=FALSE)
```

4-sample test for equality of proportions without continuity correction
data: tab_cont

X-squared = 42.454, df = 3, p-value = 3.214e-09
alternative hypothesis: two.sided
sample estimates:

prop 1	prop 2	prop 3	prop 4
0.06140351	0.09649123	0.26956522	0.35344828

***Multiple comparisons after Chi² test (without p value adjustment)**

```
>pairwise.prop.test(tab_cont, p.adj = "none")
```

Pairwise comparisons using Pairwise comparison of proportions
data: tab_cont

	Cont	Thiam0.3	Thiam1
Thiam0.3	0.4613	-	-
Thiam1	**5.0e-05**	**0.0013**	-
Thiam1.5	**1.2e-07**	**6.8e-06**	0.2171

***P value adjustment method: Bonferroni**

```
>pairwise.prop.test(tab_cont, p.adj = "bonferroni")
```

Pairwise comparisons using Pairwise comparison of proportions
data: tab_cont

	Cont	Thiam0.3	Thiam1
Thiam0.3	1.0000	-	-
Thiam1	**0.0003**	**0.0079**	-
Thiam1.5	**7.5e-07**	**4.1e-05**	1.0000

Lab 6

```
> tab_cont<-table(ab1$Treat,ab1$rfid_release_min)
> prop.test(tab_cont, alternative='two.sided',conf.level=.95,correct=FALSE)
```

4-sample test for equality of proportions without continuity correction

data: tab_cont
X-squared = 43.956, df = 3, p-value = 1.542e-09
alternative hypothesis: two.sided
sample estimates:

prop 1	prop 2	prop 3	prop 4
0.2711864	0.2881356	0.5833333	0.8076923

***Multiple comparisons after Chi² test (without p value adjustment)**

> pairwise.prop.test(tab_cont, p.adj = "none")
Pairwise comparisons using Pairwise comparison of proportions
data: tab_cont

	Cont	Thiam0.3	Thiam1
Thiam0.3	1.0000	-	-
Thiam1	**0.0011**	**0.0022**	-
Thiam1.5	**4.9e-08**	**1.3e-07**	**0.0188**

***P value adjustment method : Bonferroni**

> pairwise.prop.test(tab_cont, p.adj = "bonferroni")
Pairwise comparisons using Pairwise comparison of proportions
data: tab_cont

	Cont	Thiam0.3	Thiam1
Thiam0.3	1.0000	-	-
Thiam1	**0.0068**	**0.0132**	-
Thiam1.5	**2.9e-07**	**7.6e-07**	0.1125

2018 – HOMING DURATION

Lab 1

kruskal.test(ab$rfid_release_min~ab$Treat)
Kruskal-Wallis rank sum test
data: ab$rfid_release_min by ab$Treat
Kruskal-Wallis chi-squared = 8.0622, df = 3, p-value = 0.04474

***Multiple comparisons with Mann-Whitney test (without p value adjustment)**

pairwise.wilcox.test(ab$rfid_release_min,ab$Treat,p.adjust.method="none")
Pairwise comparisons using Wilcoxon rank sum test
data: ab$rfid_release_min and ab$Treat

	Cont	Thiam0.3	Thiam1
Thiam0.3	0.1464	-	-
Thiam1	0.0945	**0.0059**	-
Thiam1.5	0.8900	0.5501	0.2958

***P value adjustment method : Bonferroni**

> pairwise.wilcox.test(ab$rfid_release_min,ab$Treat,p.adjust.method="bonferroni")
Pairwise comparisons using Wilcoxon rank sum test
data: ab$rfid_release_min and ab$Treat

	Cont	Thiam0.3	Thiam1
Thiam0.3	0.879	-	-
Thiam1	0.567	**0.036**	-
Thiam1.5	1.000	1.000	1.000

Lab 2

kruskal.test(ab$rfid_release_min~ab$Treat)
Kruskal-Wallis rank sum test
data: ab$rfid_release_min by ab$Treat
Kruskal-Wallis chi-squared = 1.1436, df = 3, p-value = 0.7666

Lab 4

kruskal.test(ab$rfid_release_min~ab$Treat)
Kruskal-Wallis rank sum test
data: ab$rfid_release_min by ab$Treat
Kruskal-Wallis chi-squared = 10.404, df = 3, p-value = 0.01542

***Multiple comparisons with Mann-Whitney test (without p value adjustment)**

pairwise.wilcox.test(ab$rfid_release_min,ab$Treat,p.adjust.method="none")
Pairwise comparisons using Wilcoxon rank sum test
data: ab$rfid_release_min and ab$Treat

	Cont	Thiam0.3	Thiam1
Thiam0.3	0.140	-	-
Thiam1	0.042	**0.002**	-
Thiam1.5	0.879	0.156	0.111

***P value adjustment method : Bonferroni**

> pairwise.wilcox.test(ab$rfid_release_min,ab$Treat,p.adjust.method="bonferroni")
Pairwise comparisons using Wilcoxon rank sum test
data: ab$rfid_release_min and ab$Treat

	Cont	Thiam0.3	Thiam1
Thiam0.3	0.842	-	-
Thiam1	0.250	**0.012**	-
Thiam1.5	1.000	0.937	0.665

Lab 6

kruskal.test(ab$rfid_release_min~ab$Treat)
Kruskal-Wallis rank sum test
data: ab$rfid_release_min by ab$Treat
Kruskal-Wallis chi-squared = 2.9517, df = 3, p-value = 0.3991

Lab 8

kruskal.test(ab$rfid_release_min~ab$Treat)
Kruskal-Wallis rank sum test
data: ab$rfid_release_min by ab$Treat
Kruskal-Wallis chi-squared = 13.566, df = 3, p-value = 0.003559

***Multiple comparisons with Mann-Whitney test (without p value adjustment)**

pairwise.wilcox.test(ab$rfid_release_min,ab$Treat,p.adjust.method="none")
Pairwise comparisons using Wilcoxon rank sum test
data: ab$rfid_release_min and ab$Treat

	Cont	Thiam0.3	Thiam1
Thiam0.3	0.39792	-	-
Thiam1	0.50530	0.91868	-
Thiam1.5	**0.01275**	**0.00076**	**0.00170**

***P value adjustment method: Bonferroni**

> pairwise.wilcox.test(ab$rfid_release_min,ab$Treat,p.adjust.method="bonferroni")
Pairwise comparisons using Wilcoxon rank sum test
data: ab$rfid_release_min and ab$Treat

	Cont	Thiam0.3	Thiam1
Thiam0.3	1.0000	-	-
Thiam1	1.0000	1.0000	-
Thiam1.5	0.0765	**0.0046**	**0.0102**

2019 – HOMING DURATION

Lab 1

kruskal.test(ab$rfid_release_min~ab$Treat)
Kruskal-Wallis rank sum test
data: ab$rfid_release_min by ab$Treat
Kruskal-Wallis chi-squared = 2.7563, df = 3, p-value = 0.4307

Lab 2

kruskal.test(ab$rfid_release_min~ab$Treat)
Kruskal-Wallis rank sum test
data: ab$rfid_release_min by ab$Treat
Kruskal-Wallis chi-squared = 6.0721, df = 3, p-value = 0.1082

Lab 3

kruskal.test(ab$rfid_release_min~ab$Treat)
Kruskal-Wallis rank sum test
data: ab$rfid_release_min by ab$Treat
Kruskal-Wallis chi-squared = 9.905, df = 3, p-value = 0.01939

***Multiple comparisons with Mann-Whitney test (without p value adjustment)**

pairwise.wilcox.test(ab$rfid_release_min,ab$Treat,p.adjust.method="none")
Pairwise comparisons using Wilcoxon rank sum test

data: ab$rfid_release_min and ab$Treat

	Cont	Thiam0.3	Thiam1
Thiam0.3	0.051	-	-
Thiam1	**0.011**	0.411	-
Thiam1.5	**0.016**	0.241	0.473

***P value adjustment method: Bonferroni**

> pairwise.wilcox.test(ab$rfid_release_min,ab$Treat,p.adjust.method="bonferroni")
Pairwise comparisons using Wilcoxon rank sum test
data: ab$rfid_release_min and ab$Treat

	Cont	Thiam0.3	Thiam1
Thiam0.3	0.308	-	-
Thiam1	0.068	1.000	-
Thiam1.5	0.098	1.000	1.000

Lab 4

kruskal.test(ab$rfid_release_min~ab$Treat)
Kruskal-Wallis rank sum test
data: ab$rfid_release_min by ab$Treat
Kruskal-Wallis chi-squared = 4.9276, df = 3, p-value = 0.1772

Lab 5

kruskal.test(ab$rfid_release_min~ab$Treat)
Kruskal-Wallis rank sum test
data: ab$rfid_release_min by ab$Treat
Kruskal-Wallis chi-squared = 3.4626, df = 3, p-value = 0.3256

Lab 6

kruskal.test(ab$rfid_release_min~ab$Treat)
Kruskal-Wallis rank sum test
data: ab$rfid_release_min by ab$Treat
Kruskal-Wallis chi-squared = 5.9733, df = 3, p-value = 0.1129

APPENDIX 8

Assessment of Varroa infestation of the colonies for the ring test 2018 and 2019

A) 2018

Lab	Run	Sample date	Number of varroas per sample	Number of bees per sample	Number of varroas per 100 bees
1	1	14/06/2018	0	232,5	0,0
1	2	15/06/2018	0	234,2	0,0
1	3	18/06/2018	1	345,1	0,3
2	1	24/05/2018	2	335,7	0,6
2	2	31/08/2018	3	731,1	0,4
2	3	31/08/2018	2	295,5	0,7
3	1	27/07/2018	16	833,1	1,9
3	2	15/08/2018	3	672,3	0,4
3	3	24/08/2018	27	402,3	6,7
4	1	03/07/2018	3	346,4	0,9
4	2	10/07/2018	0	592,9	0,0
4	3	19/07/2018	19	537,9	3,5
5	1	26/06/2018	7	187,4	3,7
5	2	26/06/2018	4	217,9	1,8
5	**3**	26/06/2018	3	139,7	2,1
6	1	14/08/2018	12	431,9	2,8
6	2	21/08/2018	11	512,6	2,1
6	3	27/09/2018	10	279,6	3,6
7	1	27/08/2018	10	396,6	2,5
7	2	03/09/2018	18	222,3	8,1
7	3	14/09/2018	0	285,7	0,0
8	1	29/05/2018	0	356,9	0,0
8	2	04/06/2018	0	411,1	0,0
8	3	04/06/2018	0	423,4	0,0

B) 2019

Lab	Run	Sample date	Number of varroas per sample	Number of bees per sample	Number of varroas per 100 bees
1	1	13/06/2019	0	807,1	0,0
1	2	13/06/2019	0	564,3	0,0
1	3	13/06/2019	4	835,7	0,5
2	1	15/05/2019	0	278,6	0,0
2	2	15/05/2019	2	292,9	0,7
2	3	15/05/2019	0	335,7	0,0
3	1	16/06/2019	0	178,6	0,0
3	2	06/06/2019	0	178,6	0,0
3	3	12/06/2019	0	150,0	0,0
4	1	10/07/2019	62	814,3	7,6
4	2	17/07/2019	24	414,3	5,8
5	1	18/06/2019	0	500,0	0,0
5	2	21/06/2019	1	407,1	0,2
5	3	08/08/2019	7	321,4	2,2
6	1	27/06/2019	9	271,4	3,3
6	2	01/08/2019	12	350,0	3,4
6	3	08/08/2019	8	300,0	2,7
7	1	20/06/2019	0	207,1	0,0
7	2	20/06/2019	0	228,6	0,0
7	3	20/06/2019	0	157,1	0,0
8	1	05/08/2019	0	221,4	0,0
8	2	05/08/2019	0	464,3	0,0
8	3	05/08/2019	1	385,7	0,3

Landscape description (number of linears) for the ring test 2018 and 2019

A) 2018

Lab	Line	Hedge	River	Orchad	Building	Road	TOTAL
1	1	1	0	0	13	7	76
1	2	0	0	0	21	11	
1	3	0	0	0	14	9	
2	1	4	0	0	1	4	33
2	2	5	0	0	3	6	
2	3	6	0	0	1	3	
3	1	3	0	0	4	4	50
3	2	1	0	0	9	6	
3	3	1	0	0	16	6	
4	1	6	0	0	0	5	32
4	2	7	0	0	0	4	
4	3	7	0	0	2	1	
5	1	0	0	0	0	0	13
5	2	2	0	0	0	0	
5	3	4	0	0	0	0	
6	1	3	0	0	3	3	22
6	2	2	0	0	3	3	
6	3	2	0	0	1	2	
7	1	0	0	0	0	0	6
7	2	2	0	0	0	0	
7	3	4	0	0	0	0	
8	1	2	1	0	8	2	38
8	2	3	1	0	5	1	
8	3	3	0	0	9	3	

Hedge : Cypress, wooden hedge…

B) 2019

Lab	Line	Hedge	River	Orchad	Building	Road	TOTAL
1	1	6	0	0	0	5	32
1	2	7	0	0	0	4	
1	3	7	0	0	2	1	
2	1	4	0	0	1	4	33
2	2	5	0	0	3	6	
2	3	6	0	0	1	3	
3	1	1	0	0	13	7	76
3	2	0	0	0	21	11	
3	3	0	0	0	14	9	
4	1	3	0	0	4	4	52
4	2	1	0	0	9	6	
4	3	1	0	0	17	7	
5	1	5	0	0	2	3	23
5	2	2	0	0	2	3	
5	3	3	0	0	1	2	
6	1	1	0	0	0	3	21
6	2	3	0	0	1	3	
6	3	6	0	0	2	2	
7	1	5	0	0	2	4	43
7	2	4	0	0	5	3	
7	3	7	0	0	10	3	
8	1	0	0	0	1	4	19
8	2	2	0	0	1	6	
8	3	2	0	0	0	3	

Hedge : Cypress, wooden hedge…

APPENDIX 9

Detail of the generalized linear mixed effect models (GLMMs) performed on valid test runs to assess the effect of thiamethoxam dose, Varroa, temperature (punctual temperature at the release time) and landscape (number of linears) parameters as well as their interactions on honeybee homing success in 2018 and 2019

2018

Retour => Homing
Doser => Dose
Tempr => temperature
VarroaLg10r => Varroa Log 10 tranformed
Land Lg10 => Landscape Log 10 transformed

Step 1:

```
>succes2<glmer(Retour~Doser*Tempr+Doser*VarroaLg10r+Doser*LandLg10r+(1|Sitef)+(1|Sitef:
Hive), family=binomial, data=res1, na.action="na.fail")
> summary(succes2)

Generalized linear mixed model fit by maximum likelihood (Laplace Approximation) [glmerMod]
 Family: binomial  ( logit )
Formula: Retour ~ Doser * Tempr + Doser * VarroaLg10r + Doser * LandLg10r +
    (1 | Sitef) + (1 | Sitef:Hive)
   Data: res1

     AIC      BIC   logLik deviance df.resid
  2254.1   2310.6  -1117.1   2234.1     2072

Scaled residuals:
    Min      1Q  Median      3Q     Max
-5.0857 -0.7885  0.4227  0.6175  4.2456

Random effects:
 Groups     Name        Variance Std.Dev.
 Sitef:Hive (Intercept) 0.1369   0.3700
 Sitef      (Intercept) 0.2126   0.4611
Number of obs: 2082, groups:  Sitef:Hive, 17; Sitef, 7

Fixed effects:
              Estimate Std. Error z value Pr(>|z|)
(Intercept)     0.7861     0.6790   1.158  0.24701
Doser          -0.4952     0.6979  -0.710  0.47799
Tempr           0.7145     0.6364   1.123  0.26157
VarroaLg10r    -0.9453     0.4835  -1.955  0.05057 .
LandLg10r       1.2388     0.8304   1.492  0.13576
```

```
Doser:Tempr       -1.0818     0.6840  -1.582  0.11375
Doser:VarroaLg10r  -1.6023     0.4973  -3.222  0.00127 **
Doser:LandLg10r    -1.4844     0.7697  -1.929  0.05378 .
---
Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1

Correlation of Fixed Effects:
            (Intr) Doser  Tempr  VrrL10 LndL10 Dsr:Tm D:VL10
Doser       -0.365
Tempr       -0.550  0.226
VarroaLg10r -0.249  0.153 -0.190
LandLg10r   -0.847  0.287  0.260  0.103
Doser:Tempr  0.207 -0.624 -0.386  0.026 -0.094
Dsr:VrrLg10  0.158 -0.507  0.006 -0.368 -0.073  0.116
Dsr:LndLg10  0.326 -0.855 -0.123 -0.076 -0.354  0.320  0.295
```

Step 2:

```
> model.set <- dredge(succes2,rank="AIC")
Fixed term is "(Intercept)"
Warning messages:
1: In checkConv(attr(opt, "derivs"), opt$par, ctrl = control$checkConv,  :
  Model failed to converge with max|grad| = 0.00193754 (tol = 0.001, component 1)
2: In checkConv(attr(opt, "derivs"), opt$par, ctrl = control$checkConv,  :
  Model failed to converge with max|grad| = 0.00297695 (tol = 0.001, component 1)

> model.set
Global model call: glmer(formula = Retour ~ Doser * Tempr + Doser * VarroaLg10r +
    Doser * LandLg10r + (1 | Sitef) + (1 | Sitef:Hive), data = res1,
    family = binomial, na.action = "na.fail")
---
Model selection table
     (Int)     Dsr    LL1       Tmp     VL1 Dsr:LL1 Dsr:Tmp Dsr:VL1 df
74   1.8460 -1.9090                -1.0010                -1.345  6
92   1.2030 -1.1720 1.0070          -0.8762 -1.1070         -1.545  8
76   1.4940 -1.9080 0.5675          -0.9460                -1.343  7
78   1.7660 -1.9130         0.200900 -1.0330                -1.337  7
128  0.7861 -0.4951 1.2380  0.714600 -0.9454 -1.4850 -1.0820  -1.602 10
110  1.6760 -1.6440         0.452100 -1.0630         -0.6856  -1.325  8
96   1.0110 -1.1760 1.1170  0.322200 -0.9243 -1.1070         -1.532  9
80   1.3020 -1.9120 0.6784  0.321800 -0.9932                -1.330  8
112  1.2230 -1.6470 0.6633  0.568200 -1.0230         -0.6766  -1.319  9
10   2.0630 -2.4430                -1.5010                       5
12   1.6970 -2.4410 0.5967          -1.4490                       6
14   1.9470 -2.4440         0.283400 -1.5420                       6
```

46	1.8480	-2.1580	0.552800	-1.5670	-0.7144		7	
16	1.4590	-2.4410	0.7316	0.398800	-1.5040		7	
28	1.5800	-2.1610	0.7938	-1.4500	-0.4735		7	
48	1.3690	-2.1580	0.7162	0.664000	-1.5280	-0.7069	8	
32	1.3380	-2.1580	0.9328	0.404200	-1.5060	-0.4799	8	
64	1.1600	-1.6490	1.0250	0.737000	-1.5390	-0.7466	-0.8769	9
2	1.5450	-2.4360					4	
4	0.9799	-2.4340	0.9848				5	
6	1.6070	-2.4360	-0.138700				5	
20	0.8644	-2.1520	1.1800	-0.4776			6	
38	1.5140	-2.1810	0.092890	-0.6319			6	
8	0.9774	-2.4340	0.9861	0.003957			6	
40	0.8884	-2.1800	0.9785	0.234900	-0.6281		7	
24	0.8601	-2.1520	1.1830	0.006585	-0.4776		7	
56	0.6909	-1.6930	1.2700	0.297800	-0.7161	-0.7890	8	
9	1.1140		-1.4580				4	
11	0.6693		0.6805	-1.3590			5	
13	1.0650		0.113700	-1.4680			5	
15	0.4734		0.7955	0.305700	-1.3850		6	
1	0.5902						3	
3	-0.0531		1.1130				4	
5	0.6539		-0.140800				4	
7	-0.0800		1.1280	0.041650			5	

	logLik	AIC	delta	weight
74	-1119.920	2251.8	0.00	0.267
92	-1118.492	2253.0	1.14	0.151
76	-1119.629	2253.3	1.42	0.131
78	-1119.864	2253.7	1.89	0.104
128	-1117.072	2254.1	2.30	0.084
110	-1119.285	2254.6	2.73	0.068
96	-1118.344	2254.7	2.85	0.064
80	-1119.482	2255.0	3.12	0.056
112	-1118.917	2255.8	3.99	0.036
10	-1124.022	2258.0	6.20	0.012
12	-1123.726	2259.5	7.61	0.006
14	-1123.911	2259.8	7.98	0.005
46	-1123.244	2260.5	8.65	0.004
16	-1123.503	2261.0	9.16	0.003
28	-1123.513	2261.0	9.19	0.003
48	-1122.848	2261.7	9.86	0.002
32	-1123.283	2262.6	10.73	0.001
64	-1122.352	2262.7	10.86	0.001
2	-1128.191	2264.4	12.54	0.001

4 -1127.750 2265.5 13.66 0.000
6 -1128.173 2266.3 14.50 0.000
20 -1127.532 2267.1 15.22 0.000
38 -1127.644 2267.3 15.45 0.000
8 -1127.750 2267.5 15.66 0.000
40 -1127.227 2268.5 16.61 0.000
24 -1127.532 2269.1 17.22 0.000
56 -1126.768 2269.5 17.70 0.000
9 -1229.200 2466.4 214.56 0.000
11 -1228.720 2467.4 215.60 0.000
13 -1229.183 2468.4 216.53 0.000
15 -1228.596 2469.2 217.35 0.000
1 -1233.002 2472.0 220.16 0.000
3 -1232.332 2472.7 220.82 0.000
5 -1232.984 2474.0 222.13 0.000
7 -1232.331 2474.7 222.82 0.000
Models ranked by AIC(x)
Random terms (all models):
'1 | Sitef', '1 | Sitef:Hive'

Step 3:

```
> top.model <- get.models(model.set, subset=cumsum(weight)<=0.95)
Warning message:
In checkConv(attr(opt, "derivs"), opt$par, ctrl = control$checkConv,  :
  Model failed to converge with max|grad| = 0.00297695 (tol = 0.001, component 1)

> summary(top.model)
      Length Class     Mode
74  1       glmerMod S4
92  1       glmerMod S4
76  1       glmerMod S4
78  1       glmerMod S4
128 1       glmerMod S4
110 1       glmerMod S4
96  1       glmerMod S4
80  1       glmerMod S4
```

```
> mod.avg <- model.avg(top.model)
> summary(mod.avg)

CCall:
model.avg(object = top.model)

Component model call:
glmer(formula = Retour ~ <8 unique rhs>, data = res1, family =
    binomial, na.action = na.fail)

Component models:
        df   logLik     AIC delta weight
147      6 -1119.92 2251.84  0.00   0.29
12457    8 -1118.49 2252.98  1.14   0.16
1247     7 -1119.63 2253.26  1.42   0.14
1347     7 -1119.86 2253.73  1.89   0.11
1234567 10 -1117.07 2254.14  2.30   0.09
13467    8 -1119.28 2254.57  2.73   0.07
123457   9 -1118.34 2254.69  2.85   0.07
12347    8 -1119.48 2254.96  3.12   0.06

Term codes:
          Doser        LandLg10r            Tempr       VarroaLg10r
              1                2                3                4
 Doser:LandLg10r      Doser:Tempr Doser:VarroaLg10r
              5                6                7
```

Model-averaged coefficients:

(full average)

	Estimate	Std. Error	Adjusted SE	z value	Pr(>|z|)	
(Intercept)	1.4824	0.6032	0.6034	2.457	0.01402	*
Doser	-1.5903	0.6103	0.6105	2.605	0.00919	**
VarroaLg10r	-0.9703	0.4857	0.4859	1.997	0.04586	*
Doser:VarroaLg10r	-1.4103	0.4901	0.4904	2.876	0.00403	**
LandLg10r	0.4759	0.7532	0.7534	0.632	0.52763	
Doser:LandLg10r	-0.3924	0.7137	0.7139	0.550	0.58254	
Tempr	0.1628	0.4526	0.4528	0.360	0.71916	
Doser:Tempr	-0.1491	0.4378	0.4379	0.340	0.73355	

(conditional average)

	Estimate	Std. Error	Adjusted SE	z value	Pr(>|z|)	
(Intercept)	1.4824	0.6032	0.6034	2.457	0.01402	*
Doser	-1.5903	0.6103	0.6105	2.605	0.00919	**
VarroaLg10r	-0.9703	0.4857	0.4859	1.997	0.04586	*
Doser:VarroaLg10r	-1.4103	0.4901	0.4904	2.876	0.00403	**
LandLg10r	0.9051	0.8309	0.8313	1.089	0.27630	
Doser:LandLg10r	-1.2134	0.7609	0.7613	1.594	0.11099	
Tempr	0.4001	0.6391	0.6394	0.626	0.53148	
Doser:Tempr	-0.9047	0.6927	0.6930	1.305	0.19176	

Signif. codes: 0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1

2019

Retour => Homing
Doser => Dose
Tempr => temperature
VarroaLg10r => Varroa Log 10 tranformed
Land Lg10 => Landscape Log 10 transformed

Step 1:

>succes2<glmer(Retour~Doser*Tempr+Doser*VarroaLg10r+Doser*LandLg10r+(1|Sitef)+(1|Sitef:Hive), family=binomial, data=res1, na.action="na.fail")
Warning message:
In checkConv(attr(opt, "derivs"), opt$par, ctrl = control$checkConv, :
Model failed to converge with max|grad| = 0.00139202 (tol = 0.001, component 1)
> summary(succes2)

Generalized linear mixed model fit by maximum likelihood (Laplace Approximation) [glmerMod]
Family: binomial (logit)
Formula: Retour ~ Doser * Tempr + Doser * VarroaLg10r + Doser * LandLg10r + (1 | Sitef) + (1 | Sitef:Hive)
Data: res1

AIC	BIC	logLik	deviance	df.resid
2194.9	2250.9	-1087.4	2174.9	1993

```
Scaled residuals:
    Min      1Q  Median      3Q     Max
-4.1763 -0.7779  0.3668  0.6667  2.6469

Random effects:
 Groups     Name        Variance Std.Dev.
 Sitef:Hive (Intercept) 0.4875   0.6982
 Sitef      (Intercept) 0.1224   0.3499
Number of obs: 2003, groups:  Sitef:Hive, 16; Sitef, 6

Fixed effects:
                 Estimate Std. Error z value Pr(>|z|)
(Intercept)       2.03952    0.55062   3.704 0.000212 ***
Doser            -2.97143    0.40634  -7.313 2.62e-13 ***
Tempr             0.33249    1.01862   0.326 0.744116
VarroaLg10r      -0.86152    0.71659  -1.202 0.229266
LandLg10r        -0.12341    0.73049  -0.169 0.865843
Doser:Tempr      -0.31852    0.78174  -0.407 0.683679
Doser:VarroaLg10r  1.08286    0.45878   2.360 0.018260 *
Doser:LandLg10r    0.08873    0.49750   0.178 0.858447
---
Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1

Correlation of Fixed Effects:
            (Intr) Doser  Tempr  VrrL10 LndL10 Dsr:Tm D:VL10
Doser       -0.388
Tempr       -0.570  0.227
VarroaLg10r -0.424  0.210 -0.016
LandLg10r   -0.671  0.233  0.191  0.087
Doser:Tempr  0.227 -0.585 -0.346 -0.096 -0.070
Dsr:VrrLg10  0.233 -0.559 -0.111 -0.321 -0.060  0.220
Dsr:LndLg10  0.246 -0.648 -0.058 -0.070 -0.338  0.163  0.103
convergence code: 0
Model failed to converge with max|grad| = 0.00139202 (tol = 0.001, component 1)
```

<u>Step 2</u> :

```
> model.set <- dredge(succes2,rank="AIC")
Fixed term is "(Intercept)"
> model.set

Global model call: glmer(formula = Retour ~ Doser * Tempr + Doser * VarroaLg10r +
    Doser * LandLg10r + (1 | Sitef) + (1 | Sitef:Hive), data = res1,
    family = binomial, na.action = "na.fail")
---
```

Model selection table

	(Int)	Dsr	LL1	Tmp	VL1	Dsr:LL1	Dsr:Tmp	Dsr:VL1	df	logLik	AIC	delta	weight
74	2.0730	-3.015		-0.8679			1.120		6	-1087.583	2187.2	0.00	0.300
78	2.0200	-3.012		0.2136	-0.8746			1.116	7	-1087.557	2189.1	1.95	0.113
76	2.1200	-3.015	-0.11040		-0.8754			1.121	7	-1087.569	2189.1	1.97	0.112
2	1.7950	-2.624							4	-1090.798	2189.6	2.43	0.089
110	1.9750	-2.924		0.3662	-0.8470		-0.3427	1.073	8	-1087.457	2190.9	3.75	0.046
92	2.1490	-3.071	-0.17060		-0.8848	0.12220		1.128	8	-1087.539	2191.1	3.91	0.042
80	2.0610	-3.013	-0.08386	0.1904	-0.8796			1.117	8	-1087.549	2191.1	3.93	0.042
10	1.8830	-2.623		-0.3165					5	-1090.675			
6	1.7230	-2.623		0.2735					5	-1090.750			
4	1.8170	-2.624	-0.05385						5	-1090.794			
38	1.6540	-2.472		0.5775			-0.7072		6	-1090.308			
112	2.0150	-2.924	-0.07886	0.3437	-0.8518		-0.3413	1.074	9	-1087.451			
96	2.0900	-3.069	-0.14390	0.1898	-0.8891	0.12180		1.125	9	-1087.519			
14	1.8120	-2.621		0.2884	-0.3293				6	-1090.625			
12	1.9170	-2.623	-0.08091		-0.3218				6	-1090.668			
8	1.7310	-2.623	-0.01611	0.2693					6	-1090.749			
20	1.8190	-2.629	-0.05963			0.01210			6	-1090.794			
46	1.7400	-2.473		0.5903	-0.3139		-0.6974		7	-1090.195			
40	1.6590	-2.472	-0.01010	0.5748			-0.7071		7	-1090.308			
128	2.0390	-2.971	-0.12300	0.3333	-0.8606	0.08842	-0.3189	1.083	10	-1087.435			
16	1.8330	-2.621	-0.04222	0.2774	-0.3315				7	-1090.623			
28	1.9220	-2.632	-0.09066		-0.3227	0.02012			7	-1090.667			
24	1.7330	-2.628	-0.02166	0.2692		0.01158			7	-1090.749			
48	1.7570	-2.473	-0.03456	0.5810	-0.3156		-0.6969		8	-1090.194			
56	1.6460	-2.444	0.01815	0.5809		-0.05823	-0.7206		8	-1090.301			
32	1.8370	-2.630	-0.05185	0.2774	-0.3326	0.01982			8	-1090.622			
64	1.7450	-2.449	-0.01058	0.5858	-0.3128	-0.04922	-0.7084		9	-1090.189			
1	0.6428								3	-1224.796			
5	0.4927			0.5819					4	-1224.472			
9	0.7598			-0.4126					4	-1224.510			
3	0.7152		-0.17380						4	-1224.747			
13	0.6145			0.6012	-0.4354				5	-1224.184			
11	0.8508		-0.21320		-0.4233				5	-1224.439			
7	0.5417		-0.10450	0.5594					5	-1224.456			
15	0.6819		-0.14150	0.5699	-0.4402				6	-1224.157			

10 2191.4   4.18 0.037
6   2191.5   4.33 0.034
4   2191.6   4.42 0.033
38 2192.6   5.45 0.020
112 2192.9   5.74 0.017
96 2193.0   5.87 0.016
14 2193.2   6.08 0.014
12 2193.3   6.17 0.014
8   2193.5   6.33 0.013
20 2193.6   6.42 0.012
46 2194.4   7.22 0.008
40 2194.6   7.45 0.007
128 2194.9   7.70 0.006
16 2195.2   8.08 0.005
28 2195.3   8.17 0.005
24 2195.5   8.33 0.005
48 2196.4   9.22 0.003
56 2196.6   9.43 0.003
32 2197.2 10.08 0.002
64 2198.4 11.21 0.001
1   2455.6 268.43 0.000
5   2456.9 269.78 0.000
9   2457.0 269.85 0.000
3   2457.5 270.33 0.000
13 2458.4 271.20 0.000
11 2458.9 271.71 0.000
7   2458.9 271.74 0.000
15 2460.3 273.15 0.000
Models ranked by AIC(x)
Random terms (all models):
'1 | Sitef', '1 | Sitef:Hive'

Step 3 :

```
> top.model <- get.models(model.set, subset=cumsum(weight)<=0.95)
> summary(top.model)
```

```
   Length Class    Mode
74  1      glmerMod S4
78  1      glmerMod S4
76  1      glmerMod S4
2   1      glmerMod S4
110 1      glmerMod S4
92  1      glmerMod S4
80  1      glmerMod S4
10  1      glmerMod S4
6   1      glmerMod S4
4   1      glmerMod S4
38  1      glmerMod S4
112 1      glmerMod S4
96  1      glmerMod S4
14  1      glmerMod S4
12  1      glmerMod S4
8   1      glmerMod S4

> mod.avg <- model.avg(top.model)
> summary(mod.avg)

Call:
model.avg(object = top.model)

Component model call:
glmer(formula = Retour ~ <16 unique rhs>, data = res1, family =
    binomial, na.action = na.fail)

Component models:
      df   logLik     AIC delta weight
147    6 -1087.58 2187.17  0.00   0.32
1347   7 -1087.56 2189.11  1.95   0.12
1247   7 -1087.57 2189.14  1.97   0.12
1      4 -1090.80 2189.60  2.43   0.09
13467  8 -1087.46 2190.91  3.75   0.05
12457  8 -1087.54 2191.08  3.91   0.05
12347  8 -1087.55 2191.10  3.93   0.04
14     5 -1090.68 2191.35  4.18   0.04
13     5 -1090.75 2191.50  4.33   0.04
12     5 -1090.79 2191.59  4.42   0.03
136    6 -1090.31 2192.62  5.45   0.02
123467 9 -1087.45 2192.90  5.74   0.02
123457 9 -1087.52 2193.04  5.87   0.02
134    6 -1090.62 2193.25  6.08   0.02
```

```
124     6 -1090.67 2193.34  6.17   0.01
123     6 -1090.75 2193.50  6.33   0.01

Term codes:
          Doser        LandLg10r           Tempr       VarroaLg10r
              1                2               3                4
 Doser:LandLg10r      Doser:Tempr Doser:VarroaLg10r
              5                6               7

Model-averaged coefficients:
(full average)
                  Estimate Std. Error Adjusted SE z value Pr(>|z|)
(Intercept)       1.994445   0.394771    0.394984   5.049    4e-07 ***
Doser            -2.903423   0.297535    0.297649   9.755   <2e-16 ***
VarroaLg10r      -0.658406   0.718615    0.718943   0.916    0.360
Doser:VarroaLg10r 0.815219   0.625921    0.626064   1.302    0.193
Tempr             0.091532   0.564259    0.564580   0.162    0.871
LandLg10r        -0.031670   0.375427    0.375650   0.084    0.933
Doser:Tempr      -0.037679   0.262054    0.262174   0.144    0.886
Doser:LandLg10r   0.007564   0.126024    0.126097   0.060    0.952

(conditional average)
                  Estimate Std. Error Adjusted SE z value Pr(>|z|)
(Intercept)        1.9944     0.3948      0.3950   5.049    4e-07 ***
Doser             -2.9034     0.2975      0.2976   9.755   <2e-16 ***
VarroaLg10r       -0.8231     0.7142      0.7146   1.152   0.2493
Doser:VarroaLg10r  1.1155     0.4485      0.4487   2.486   0.0129 *
Tempr              0.2735     0.9495      0.9501   0.288   0.7735
LandLg10r         -0.1034     0.6729      0.6733   0.154   0.8779
Doser:Tempr       -0.4290     0.7836      0.7841   0.547   0.5842
Doser:LandLg10r    0.1221     0.4923      0.4926   0.248   0.8042
---
Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1
```