Lack of Effects On Bumblebee (*Bombus Terrestris*) Colony Development And Drone And Queen Production Due To The Insecticide Chlorantraniliprole

Axel Dinter (axel.dinter@fmc.com)
FMC Agricultural Solutions https://orcid.org/0000-0002-8069-2566
Olaf Klein
Eurofins Agroscience Services Ecotox GmbH
Lea Franke
Eurofins Agroscience Services Ecotox GmbH

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Abstract

The aim of the study was to investigate the potential impact of the insecticide chlorantraniliprole on queen-right bumblebee (*Bombus terrestris*) colonies under semi-field conditions in *Phacelia tanacetifolia*. The *P. tanacetifolia* crop was grown in soil treated with modelled worst-case 20-year plateau concentration of chlorantraniliprole in the top 20 cm of soil (equivalent to 0.088 mg a.s./kg). Additionally, two chlorantraniliprole spray applications at 60 g a.s./ha were made. In treatment T1 both spray applications took place before *P. tanacetifolia* flowering at growth stages BBCH 51–55 and BBCH 55–59. In T2 one spray application was conducted before *P. tanacetifolia* flowering at BBCH 55–59 and one application during *P. tanacetifolia* flowering and during daily bee flight at BBCH 61–62. The application in the control (C) and reference item treatment (R) (400 g dimethoate a.s./ha) was carried out during full *P. tanacetifolia* flowering and bumblebee flight. The bumblebee colonies were exposed to the treated flowering *P. tanacetifolia* crop for 20 days in the tunnels and afterwards the colonies were kept on a monitoring site. Results of this study indicate no significant differences between the chlorantraniliprole groups T1 and T2 and the control regarding all parameters assessed (i.e. adult and larval mortality, flight activity at the hive entrance, colony weight development, condition of the colonies and production of young queens and males). Overall, no effects of chlorantraniliprole on *B. terrestris* colonies including queen/male production, adult and larval survival, colony development and forager flight activity were found in this worst-case exposure set-up.

Introduction

Next to honeybees, bumblebees are important pollinators for many wild plants and agricultural crops (Klein et al. 2007). To secure the high and increasing global demand for food – including those requiring pollinating bees – plant protection products are regularly needed and used to guarantee high crop quality and high crop yield. The potential yield decline – due to partial (20 percent) or complete (100 percent) conversion from conventional farming (including use of chemical plant protection products) to organic farming would require about 815000 hectares or 6.5 million hectares, respectively, of currently unused arable land as outlined exemplarily for Germany by Noleppa (2016) and would significantly reduce natural biodiversity including those of pollinating insects.

To investigate the potential impact of plant protection products on bumblebees, different testing and risk assessment approaches have been proposed over the last decades (Thompson (2001), van der Steen (2001)). Worst-case laboratory studies with easily available life stages (e.g. adult bumblebee worker bees) are a starting point to investigate the intrinsic sensitivity of bumblebees to plant protection products. Laboratory tests with artificial colonies of a couple of worker bees of which one worker bee assumes the function of a pseudo-queen enabling such colonies to produce drones may be used to study the impact of plant protections products on colony development. The results of such studies gain insight into the potential effects but may lack realistic exposure assumptions and therefore – in most cases – are unrealistically (intentionally or unintentionally) overdosed versus the actual exposure situation under field conditions.

Chlorantraniliprole is an anthranilic diamide insecticide and is registered and used in many countries worldwide (Cordova et al. 2006, Lahm et al. 2007) and has proven to have negligible effects on numerous beneficial non- target arthropod species or to have a rather low and transient impact on some beneficial species (e.g. Dinter et al. 2008, Brugger et al. 2010, Larson et al. 2012). A bumblebee greenhouse study did not indicate any negative impact on commercial bumblebee colonies for chlorantraniliprole use in tomato (Dinter et al. 2009). Also, Gradish et al. (2010) concluded on basis of research with *Bombus impatiens* that chlorantraniliprole is safe for greenhouse uses in presence of bumblebees. A worst-case laboratory chronic oral exposure study with small artificial *Bombus terrestris* colonies without a queen with constant exposure to chlorantraniliprole via pollen dosed at 0.4 to 40 mg a.s./kg over 7 weeks resulted in suppression of reproduction in worker bumblebees (Smagghe et al. 2013). In a bumblebee semi-field tunnel study with queen-right *B. terrestris* colonies with two chlorantraniliprole spray applications each made at 60 g chlorantraniliprole/ha during *P. tanacetifolia* flowering no negative impact on reproduction of bumblebees was found (Dinter and Brugger 2015).

The aim of this study was to investigate the effects of the insecticide chlorantraniliprole (using Coragen® insect control, containing 200 g chlorantraniliprole active substance/L (Chlorantraniliprole 20SC)) on the bumblebee (*Bombus terrestris* L.) under realistic worst-case semi-field conditions in *Phacelia tanacetifolia* based on general SETAC/ESCORT recommendations (BARRETT et al. 1994), EPPO Guideline No. 170 (4) (2010) and ring-test protocols from the ICPPR Non-Apis workshops (2016 and 2017). Chlorantraniliprole was incorporated into the 20 cm topsoil layer in which the *P. tanacetifolia* crop was grown and then received two further spray applications with chlorantraniliprole either during pre-flowering or during pre- and flowering period.

Materials And Methods

Study design and location

The semi-field tunnel study with *Phacelia tanacetifolia* as crop was located in Southern Germany and conducted in 2019. The trial consisted of six replicate tunnels each for the water-treated control (C) and the two chlorantraniliprole treated groups T1 and T2, and three replicate tunnels for the toxic reference (R) for biological assessments. Each tunnel covered an area of approx. 60 m² (5 m x 12 m and 3.5 m maximal height) and was covered with a net with a mesh size of approx. 1.5 mm and had one bumblebee colony per tunnel. In these tunnels linen sheets were spread along the middle of the tunnel and along the walls of the short sides for the mortality assessments (Fig. 1). Additionally, one replicate tunnel per chlorantraniliprole treated groups T1 and T2 and control treatment (C) with three bumblebee hives per tunnel were prepared for residue pollen/nectar sampling. The tunnel size was approx. 100 m². The distance from the treated tunnels T1 and T2 to the untreated control and toxic reference tunnel (C and R) was 95 m to avoid potential cross contamination. The distance between the individual tunnel replicates was 4 to 6 m.

The bumblebee trial investigated exposure via potential soil uptake (A1 spray application made onto bare soil and incorporated into 20 cm top soil before *P. tanacetifolia* sowing) ending in pollen/nectar contamination combined with an early and a late pre-flowering spray (A2 and A3) (scenario T1) or combined with one late pre-flowering spray (A3) and one spray application made during *Phacelia* flowering while the bumblebees were actively foraging during the day (A4) (worst-case exposure scenario T2) (Table 1). The first application (A1) of Chlorantraniliprole 20SC was applied (16 April 2019) to bare soil at a rate of 265.15 g a.s./ha and mixed into the 20 cm top soil before *P. tanacetifolia* seeding to achieve a modelled worst-case 20-year plateau concentration in 20 cm top soil (equivalent to 0.088 mg a.s./kg assuming a worst-case soil DT50 of 697.5 days and 2 sprays at 60 g a.s./ha with a 7-day retreatment interval). Additionally, two foliar applications of 60 g a.s./ha were conducted in T1 and T2. In T1 Chlorantraniliprole 20SC applications (A2 and A3) took place before *P. tanacetifolia* flowering with a 6-day spray interval (A2 at BBCH 51–55 and A3 at BBCH 55–59). In T2 spray applications were conducted once before *P. tanacetifolia* flowering (BBCH 55–59 (A3)) and once during *P. tanacetifolia* flowering and during daily bumblebee flight (BBCH 61–62 (A4)) with an 8-day spray interval. The application in the control (C) (water only) and reference item treatment (R) (800 g dimethoate a.s./ha) was carried out during full *P. tanacetifolia* flowering and bumblebee flight on the same day as the 2nd application of T2 (A4). All spray applications were performed at a water volume of 300 L tap water/ha.

Table 1
Detailed information on timing and application rates of the chlorantraniliprole treatments T1 and T2, toxic reference (R), and control (C)

Application number	Application date (time)	Timing	Growth stage of Phacelia crop (BBCH stage)	Treatment group	Chlorantraniliprole 20SC [g a.s./ha]		
					Target rate (nominal)	Target rate (actual)*	Analytical rate verification**
A1	16 Apr 2019	-62DBA4	bare soil spray and mixed into 20 cm topsoil layer	**T1/T2**	**265.15**	261.17	75%
A2	03 Jun 2019	-14DBA4	51–55	**T1**	**60.0**	68.1	93%
A3	09 Jun 2019	-8DBA4	55–59	**T1**	**60.0**	64.4	105%
			55–59	**T2**	**60.0**	63.2	108%
A4	17 Jun 2019 (9:53 – 11:30 am)	0DBA4	61–62	**T2**	**60.0**	67.2	104%
	17 Jun 2019 (9:40 – 10:20 am)		61–62	**C**	**0** (300 L **water**/ha)	0 (316 L water/ha)	-
	17 Jun 2019 (9:53 – 11:30 am)		61–62	**R**	**0** (800.0 g **dimethoate**/ha)	0 (811.5 g dimethoate/ha)	-

* Applied rate on basis of measured applied spray volume.

** Analytically verified rate (%) on basis of top 20 cm soil residue analysis for A1 and on basis of spray solution analysis for A2 to A4.

On 13 June 2019 the young queen right bumblebee colonies (*Bombus terrestris* L., Hymenoptera, Apidae) (origin Koppert BV) with an average of 57 worker bumblebees per colony were evaluated under laboratory conditions (initial whole colony and initial colony weight assessment). The following day the colonies were set up inside the tunnels at BBCH 59–61 on 14 June 2019 five days after application A3 and three days before application A4 (= 3DBA4). The bumblebee colonies were exposed to the treated flowering *P. tanacetifolia* crop for 20 days in the tunnels. The colonies did not receive any supplementary feeding with sugar solution during the experiment. The colonies were assessed during the flowering period for mortality (adults and larvae in the tunnels on linen sheets and inside of the hive), flight activity at the hive entrance, development of colony weight and development of the bumblebee brood. At the end of flowering of *P. tanacetifolia* (BBCH 69) the bumblebee hives were transferred to a field monitoring site and were further assessed for mortality, colony weight and production of young queens and males. The colonies were kept at the monitoring site until approx. 30–40 % of the estimated queen pupae had emerged and then were individually deep-frozen. When it was foreseeable that a colony would not reach the switch-point to produce queens/drones, it was deep-frozen earlier (i.e. all three R colonies). After deep-

freezing of all colonies, a final colony assessment was conducted to get a detailed overview of the colony brood development.

Assessment during the exposure and monitoring phase

Observation of flight activity at the entrance of the bumblebee colonies started after set-up of the colonies in the tunnels and was carried out for each of the replicate bumblebee colonies employed for biological assessments. At each assessment date during the exposure phase, the number of bumblebees entering and exiting the colony entrance within 10 minutes was counted. Assessments of flight activity were done in parallel in the treatment groups C, T1 and T2. To show that bumblebees were foraging actively on the target crop the number of foraging bumblebees carrying pollen loads when entering the hive was counted during the assessments. Additionally, once per assessment day it was checked that bumblebee foragers were seen collecting nectar and pollen on *P. tanacetifolia* flowers. During the exposure phase in the tunnels, mortality was evaluated inside the hives, in front of the hives and on the linen sheets. After the exposure phase the mortality assessments inside the hives were continued at the monitoring site. At each assessment dead adults and larvae were counted and removed. Foundress queen mortality was also noted. The area around the hives, the hive entrance (colony) and all linen sheets were checked. All dead adults and larvae were counted and removed (mortality in the tunnels). The weight development of the bumblebee colonies was determined during the confined exposure and monitoring phase. The weight serves as a benchmark for the growth of the brood nest and collecting activity of the bumblebees during the study. To calculate the weight of each bumblebee colony without the hive box, the empty hive box of each colony was weighed after the final brood assessment.

The production of young queens and males was assessed during the exposure and monitoring phase. The “switch point” was reached when first queen pupae were detected in the colonies (26 Jun to 03 Jul 2019; 9 to 16 DAA4). Before the colonies were transferred to the monitoring site queen excluder were installed in C, T1 and T2 (01 Jul 2019) to prevent young queens from leaving the hives. In the toxic reference R, no queen excluders were installed, as the hives did not develop any queen brood. As soon as first young queens emerged, the queens were collected two times per week and were transported to the laboratory on dry ice, weighed individually and stored deep-frozen until the final brood assessment. When approx. 30–40% of the estimated queen pupae (all queen pupae visible from the top during the mortality assessments (queen pupae in the lower layers within the brood nest cannot be counted without destroying the brood nest)) had emerged, the colony was deep-frozen (each hive individually). As it was foreseeable that all R colonies would not reach the switch point due to the loss of all foundress queens and only 0 to 4 alive workers per colony, they were deep-frozen on 04 Jul 2019 (at the end of the exposure phase).

For the initial brood assessment each colony was opened under red light. The adult workers were counted and taken out of the hives for weighing. The bumblebee colony was weighed, and the sugar solution was removed. After weighing of the hives and the subsequent brood assessment (determination of all brood stages), adult workers were transferred back in their hives. Colonies were also examined for malformations, predators and parasites. Before the final brood assessment, the bumblebee hives were stored deep-frozen. The adult bumblebees were counted and taken out of the hives for weighing. The different brood stages and cells for the storage of food were counted by removing them bit by bit from the colony. Colonies were also examined for malformations, predators and parasites.

Samplings for residue analysis, residue analysis and statistical analysis

On 16 April 2019 (-62DAA4) shortly after the first spray application (A1) and soil incorporation into the top 20 cm soil layer i.e. on the same day (before sowing of the Phacelia seeds), 10 soil samples (with a diameter of 5 cm) per application area for area C/R and area T1/T2 were taken from the 0–20 cm horizon. The 10 samples of each area were pooled, resulting in one mixed sample per area. The samples were chilled during transport to the freezer (on blue ice) and were subsequently stored deep frozen at ≤ -18°C within 12 h after end of sampling. Spray solution samples were taken at each application before spraying from the area T1/T2 (A1) or from one replicate (A2, A3 and A4). Samples of the spray solutions were taken

after the spray solution had been properly mixed. Samples were kept on dry ice immediately after sampling until deep-freezing, except for the sample from area T1/T2 (A1), which was transported at ambient conditions to the deep freezer within less than 30 min after the sample was taken.

Pollen and nectar from *P. tanacetifolia* flowers were sampled via forager bumblebees in the residue tunnels. In order to sample pollen and nectar for residue analysis, the forager bumblebees were captured directly at the flowers with plastic bottles containing some dry ice to narcotize the bees. Preparation of pollen loads and nectar from forager bumblebees was done directly at the field site. Nectar was collected from stomachs of several dissected bumblebees to get a minimum amount of 200 mg nectar per sample. The pollen loads from forager bumblebees were detached from the hind legs to get a minimum amount of 200 mg pollen per sample. Samples were kept on dry ice immediately after sampling until deep-freezing and were subsequently stored deep frozen at ≤ -18°C within 12 h after end of sampling.

Residue analysis of soil, spray solution, nectar and pollen samples was performed according to fully validated analytical methods according to SANCO/825/00 rev 8.1. Quantification was performed by use of LC-MS/MS detection and a limit of quantification (LOQ) of 0.0005 mg chlorantraniliprole/kg.

For the statistical analysis the statistical software program SAS Version 9.4 was used. *P. tanacetifolia* growth stages are given according to BBCH growth stages by Meier (2001).

Results

Foraging activity

After the set-up of the bumblebee colonies in the tunnels on -3DAA4 the bumblebees started to forage immediately with 0.8 to 2.8 entering and leaving bumblebees/10 min (Table 2). In all 4 treatment groups, the control C, the chlorantraniliprole groups T1 and T2 and the toxic reference R, flight activity increased until the day of application A4 with 8.0 to 12.0 bumblebees/10 min. No statistically significant differences were observed in T1, T2 and R before application A4 compared to the control. Directly after the application (0DAA4) no statistically significant differences were seen between C and T1 with 11.3 and 9.7 entering and exiting bumblebees/10 min, but flight activity was observed to be slightly but significantly lower in T2 with 6.5 bumblebees/10 min ($p \leq 0.05$, Dunnetts t-test). However, from 1DAA4 until 16DAA4 no statistically significant differences were observed between the control and the chlorantraniliprole groups T1 and T2, except for a significantly higher flight activity in T1 on 5DAA4 ($p \leq 0.05$, Dunnetts t-test). The mean flight activity in C, T1 and T2 was not statistically significant different during the whole exposure period with 10.9, 12.5 and 11.9 bumblebees/10 min, respectively. Instead, the flight activity in the toxic reference R was significantly reduced on all assessment dates from 0DAA4 until 16DAA4 ($p \leq 0.05$, pooled t-test, Satterthwaite t-test, Mann Whitney exact test).

Table 2
Mean bumblebee flight activity (Mean number of forager bees entering and leaving the hive per 10 min) in chlorantraniliprole treatment group T1 and T2, control (C) and toxic reference (R)

Date	DAA4	Flight activity (Mean number of forager bees entering and leaving the hive per 10 min)			
		C	T1	T2	R
14 Jun 2019	-3	2.0	2.8	0.8	1.3
15 Jun 2019	-2	3.5	4.3	4.5	5.3
16 Jun 2019	-1	8.0	12.0	11.3	10.0
17 Jun 2019	0	11.3	9.7	6.5* a)	2.0* b)
18 Jun 2019	1	12.0	8.3	8.2	0.0* c)
19 Jun 2019	2	12.0	11.0	12.0	0.0* c)
20 Jun 2019	3	9.0	8.2	7.8	0.0* c)
21 Jun 2019	4	5.7	9.2	4.7	0.0* c)
22 Jun 2019	5	5.3	10.7* a)	9.7	0.0* d)
23 Jun 2019	6	15.7	14.2	21.8	0.0* c)
24 Jun 2019	7	9.7	10.8	11.7	0.0* c)
26 Jun 2019	9	6.0	10.7	7.7	0.0* c)
29 Jun 2019	12	12.5	17.5	21.7	0.0* c)
01 Jul 2019	14	21.8	26.2	26.7	0.0* c)
03 Jul 2019	16	29.3	31.7	24.0	0.0* c)
Mean of tunnel phase		**10.9**	**12.5**	**11.9**	**1.2*** b)

DAA4 = days after 4th application; Mean = mean value of all tunnel replicates; STD = standard deviation of the mean of the tunnel replicates;

* statistically significant difference compared to control ($p \leq 0.05$)

a) Dunnetts t-test; b) pooled t-test; c) Satterthwaite t-test; d) Mann Whitney exact

Adult and larval mortality

During the initial exposure phase from − 3DAA4 to -1DAA4 the adult and larval mortality determined inside the hive was low and not statistically significant different in the control, the chlorantraniliprole groups T1 and T2 and the toxic reference R with maximally 0.3 dead workers/day and 0.3 larvae/day (Table 3). Adult and larval mortality observed in the hives was generally low and not statistically significant different in T1 and T2 compared to the control during the study from − 3DAA4 to 25DAA4, with maximum values of 1.7 dead workers per day in C on 18DAA4, 1.1 in T1 on 25DAA4 and 1.8 in T2 on 1DAA4. There were no statistically significant differences in mean total mortality during the exposure phase found in C, T1 and T2 with 2.5, 2.2 and 4.3 dead adult workers and 4.2, 5.8 and 4.5 dead larvae inside the colonies, respectively. In the toxic reference mortality of adult bumblebees was statistically significant higher compared to the control on all assessment days from 0DAA4 to 14DAA4 ($p \leq 0.05$, Satterthwaite t-test, Mann Whitney exact test), except for 5DAA4 and

6DAA4, with a maximum number of 22.7 dead workers on 1DAA4. The mean total mortality of workers during the exposure period was also statistically significantly higher with 94.3 dead workers in the toxic reference compared to 2.5 workers in the control ($p \leq 0.05$, Mann Whitney exact test).

Table 3
Mean number of dead in-hive bumblebee adults and larvae in chlorantraniliprole treatment group T1 and T2, control (C) and toxic reference (R)

Date	DAA4	Mean number of dead in-hive bumblebee adults and larvae							
		C		T1		T2		R	
		Adults	Larvae	Adults	Larvae	Adults	Larvae	Adults	Larvae
14 Jun 2019	-3	0.2	0.0	0.0	0.2	0.0	0.0	0.3	0.3
15 Jun 2019	-2	0.2	0.2	0.0	0.2	0.0	0.0	0.0	0.3
16 Jun 2019	-1	0.3	0.3	0.0	0.0	0.0	0.0	0.0	0.0
17 Jun 2019	0	0.0	0.3	0.0	0.5	0.0	0.0	9.0* b)	0.0
18 Jun 2019	1	0.0	0.5	0.2	1.3	1.8	0.2	22.7* b)	3.7* b)
19 Jun 2019	2	0.0	0.3	0.2	0.2	0.3	0.0	18.7* b)	3.7
20 Jun 2019	3	0.0	0.2	0.2	0.0	1.0	1.2	13.7* b)	2.0
21 Jun 2019	4	0.0	0.2	0.0	0.0	0.0	0.5	5.3* b)	6.3
22 Jun 2019	5	0.3	0.7	0.2	0.3	0.2	0.0	3.3	2.0
23 Jun 2019	6	0.2	0.5	0.0	0.2	0.0	0.2	0.7	11.0* b)
24 Jun 2019	7	0.0	0.2	0.0	0.3	0.0	0.2	2.7* b)	0.0
26 Jun 2019	9	0.0	0.0	0.1	0.0	0.1	0.0	1.8* b)	0.2
29 Jun 2019	12	0.1	0.1	0.1	0.2	0.0	0.1	1.7* b)	0.0
01 Jul 2019	14	0.3	0.1	0.1	0.1	0.1	0.3	3.2* a)	0.2
03 Jul 2019	16	0.3	0.2	0.4	0.9	0.3	0.8	1.5	0.0
Sum of tunnel phase[1)]		**2.5**	**4.2**	**2.2**	**5.8**	**4.3**	**4.5**	**94.3*** b)	**30.0**
05 Jul 2019[2)]	18	1.7	1.2	0.4	2.8	0.6	3.0	-	-
09 Jul 2019	22	0.3	1.3	0.3	1.8	0.4	3.1	-	-
12 Jul 2019	25	0.6	9.7	1.1	6.4	1.1	11.8	-	-

DAA4 = days after 4th application; Mean = mean value of all tunnel replicates; STD = standard deviation of the tunnel replicates; - = no data available (all hives deep-frozen)

Mean mortality is only shown for days, on which values for all replicates were available (between 29 and 36 DAA4 individual replicates were deep-frozen).

[1)] Calculated as mean of the total sum of dead bumblebee adults or larvae from − 3 to 16 DAA4

[2)] First assessment on monitoring site

* statistically significant difference compared to control ($p \leq 0.05$)

[a)] Satterthwaite t-test; [b)] Mann Whitney exact

Only very few dead adult and larval bumblebees were found additionally in the tunnels on the linen sheets. The sum of dead bumblebee adults and larvae found in the tunnels C, T1, T2, and R during the whole exposure phase was 0.3, 0.5, 0.2 and 3.0 bees, respectively. Only the R treatment was statistically significant different to the control ($p \leq 0.05$, Mann Whitney exact).

Colony weights

The mean bumblebee colony weights were not statistically significant different between the control and the T1 and T2 throughout the study (-3DAA4 to 25DAA4) (Table 4). Colony weights increased continuously from 1DAA4 until 25DAA4 with maximum weights of 487 g, 617 g and 596 g in the control and T1 and T2, respectively. The total weight increase from – 3DAA4 until deep-freezing of the colonies was also similar and not statistically significant different with 396 g in the control, 524 g in T1 and 488 g in T2. Colony weights in the toxic reference were similar to the control from – 3DAA4 until 1DAA4 and decreased from 2DAA4 onwards. The mean total colony weight increase from – 3DAA4 until 16DAA4 was statistically significantly lower in the toxic reference R compared to the control ($p \leq 0.05$, Satterthwaite t-test).

Table 4
Mean weight of bumblebee colonies (g ± standard deviation (STD)) in chlorantraniliprole treatment group T1 and T2, control (C) and toxic reference (R)

Date	DAA4	Mean weight of bumblebee colonies (g ± standard deviation)							
		C		T1		T2		R	
		Mean	STD	Mean	STD	Mean	STD	Mean	STD
14 Jun 2019	-3	**96**	7	**101**	13	**108**	11	**101**	20
15 Jun 2019	-2	**88**	5	**93**	11	**99**	9	**93**	17
16 Jun 2019	-1	**88**	6	**97**	13	**102**	8	**92**	14
17 Jun 2019	0	**90**	7	**106**	14	**110**	10	**92**	12
18 Jun 2019	1	**101**	9	**115**	12	**110**	9	**85**	17
19 Jun 2019	2	**113**	8	**123**	13	**113**	10	**79*** [a)]	16
20 Jun 2019	3	**124**	7	**142**	16	**132**	13	**75*** [a)]	17
21 Jun 2019	4	**141**	8	**161**	18	**149**	16	**71*** [a)]	14
22 Jun 2019	5	**155**	9	**179**	23	**164**	20	**70*** [a)]	13
23 Jun 2019	6	**157**	10	**183**	21	**164**	16	**70*** [a)]	11
24 Jun 2019	7	**178**	12	**206**	29	**190**	15	**67*** [a)]	10
26 Jun 2019	9	**209**	11	**254**	50	**238**	25	**51*** [a)]	18
29 Jun 2019	12	**270**	21	**355**	69	**336**	40	**61*** [a)]	8
01 Jul 2019	14	**338**	33	**444**	79	**440**	47	**60*** [a)]	6
03 Jul 2019	16	**391**	65	**493**	76	**491**	50	**56*** [b)]	7
05 Jul 2019[1)]	18	**399**	63	**502**	90	**498**	55	**-**	-
09 Jul 2019	22	**468**	88	**587**	106	**568**	73	**-**	-
12 Jul 2019	25	**487**	80	**617**	105	**596**	73	**-**	-

DAA4 = days after 4th application; Mean = mean value of all tunnel replicates; STD = standard deviation of the tunnel replicates; - = no assessment performed or not calculable

[1)] first assessment on monitoring site

[2)] Total weight increase per colony calculated by subtracting the first assessment date from the maximum weight measured (calculated separately for each replicate from set-up (-3 DAA4) until deep-freezing of the colony).

Mean weights are only shown for days, on which values for all replicates were available (between 29 and 36 DAA4 individual replicates were deep-frozen).

* statistically significant difference to control ($p \leq 0.05$)

[a)] pooled t-test; [b)] Satterthwaite t-test

Date	DAA4	Mean weight of bumblebee colonies (g ± standard deviation)							
		C		T1		T2		R	
		Mean	STD	Mean	STD	Mean	STD	Mean	STD
Total weight increase [g] [2)]		**396**	79	**524**	89	**488**	65	**1*** [b)]	2
DAA4 = days after 4th application; Mean = mean value of all tunnel replicates; STD = standard deviation of the tunnel replicates; - = no assessment performed or not calculable									
[1)] first assessment on monitoring site									
[2)] Total weight increase per colony calculated by subtracting the first assessment date from the maximum weight measured (calculated separately for each replicate from set-up (-3 DAA4) until deep-freezing of the colony).									
Mean weights are only shown for days, on which values for all replicates were available (between 29 and 36 DAA4 individual replicates were deep-frozen).									
* statistically significant difference to control ($p \leq 0.05$)									
[a)] pooled t-test; [b)] Satterthwaite t-test									

Colony development

At the initial brood assessment, before the bumblebee colonies were set up in the tunnels, all bumblebee colonies were queen-right and in good condition with a mean number of 57.3 workers per colony in C, 57.3 in T1, 57.2 in T2 and 56.0 in R. Additionally, all colonies showed similar strength with regard to the number of living brood stages and food storage. No statistically significant differences in the condition of the bumblebee colonies of T1, T2 and R compared to the control were observed at the initial brood assessment (Table 5). Also, at the final brood assessment no statistically significant differences in the number of the individual living or dead brood stages were found in T1 and T2 compared to the control. The total number of living adult and living brood stages were similar in the control, T1 and T2 with 99.2 living adult bees and 263.3 living brood stages in C, 153.3 and 431.8 in T1 and 135.0 and 453.5 in T2, respectively. In the toxic reference the following parameters were statistically different from the control: the number of living workers, the number of living young and old larvae (separately and the sum) and the number of dead larvae ($p \leq 0.05$, pooled t-test, Satterthwaite t-test). Also, the total number of living adult and living brood stages was significantly reduced compared to the control with 12.0 living adult bees and 63.0 living brood stages in R compared to 99.2 living adult bees and 263.3 living brood stages in C ($p \leq 0.05$, pooled t-test, Satterthwaite t-test). Foundress queen mortality was observed in one of six replicates of T2 during the exposure phase (2DAA4). The reason for the death of the foundress queen was not clear and the colony appeared to be healthy. The cause of this unexplained mortality was assumed to be natural background mortality and not treatment related. It is not unusual, that one foundress queen is lost during a study. Therefore, the foundress queen was replaced with a foundress queen from a similarly treated hive. Apart from this replicate no foundress queen mortality was observed in any of the control or chlorantraniliprole T1 and T2 colonies. In the toxic reference all foundress queens died within the first 5 days after the application.

Table 5

Summary of results of initial and final colony assessments for chlorantraniliprole treatment group T1 and T2 control (C) and toxic reference (R)

Number of adult and larval stages (Mean ± standard deviation) and Colony weight (g ± standard deviation)								
Initial colony assessment: 13 Jun 2019								
Life stage	**C**		**T1**		**T2**		**R**	
	Mean	STD	**Mean**	STD	**Mean**	STD	**Mean**	STD
Living queens	**1**	0	**1**	0	**1**	0	**1**	0
Living workers	**57.3**	7.0	**57.3**	7.6	**57.2**	9.6	**56.0**	7.2
Brood cells with eggs	**7.0**	2.1	**7.8**	2.7	**8.7**	2.3	**8.0**	1.0
Brood cells with young larvae (package)	**64.3**	16.0	**68.2**	14.4	**52.7**	12.2	**68.7**	36.0
Brood cells with old larvae (isolated)	**16.8**	10.5	**24.7**	11.5	**28.8**	6.4	**24.7**	5.9
Living pupae (worker/males)	**49.0**	8.7	**50.2**	13.9	**47.5**	13.3	**42.3**	14.5
Filled nectar cells	**30.3**	9.2	**25.2**	4.3	**27.7**	3.6	**31.7**	15.5
Colony weight [g]	**86.5**	8.4	**87.3**	14.3	**96.8**	13.9	**88.0**	18.5
Total living brood stages (eggs, larvae, pupae)	**137.2**	15.8	**150.8**	30.0	**137.7**	20.8	**143.7**	51.3
Total living stages (brood and adult bees)	**194.5**	15.4	**208.2**	28.9	**194.8**	27.0	**199.7**	57.7
Final colony assessment: 08–15 Aug 2019								
Life stage	**C**		**T1**		**T2**		**R**	
	Mean	STD	**Mean**	STD	**Mean**	STD	**Mean**	STD
Living workers	**34.7**	18.4	**45.5**	10.7	**41.7**	10.6	**11.7*** [a)]	10.3
Dead workers	**4.8**	4.2	**11.2**	8.8	**8.7**	5.2	**1.3**	1.5
Eggs	**33.2**	25.7	**82.7**	81.7	**125.5**	41.6	**15.3**	5.9
Living young larvae	**74.3**	39.4	**114.5**	52.3	**124.8**	71.0	**0.0*** [b)]	0.0
Living old larvae (isolated, worker/males)	**21.5**	11.7	**33.7**	16.8	**21.0**	3.0	**3.0*** [a)]	3.0
Sum of living larvae (worker/males)	**95.8**	48.8	**148.2**	60.6	**145.8**	69.8	**3.0*** [b)]	3.0
STD = standard deviation; - = not available								
Calculation of weights based on unrounded values								
* statistically significant difference compared to control ($p \leq 0.05$)								
[a)] pooled t-test; [b)] Satterthwaite t-test; [c)] Mann Whitney exact								

Number of adult and larval stages (Mean ± standard deviation) and Colony weight (g ± standard deviation)								
Sum of dead larvae (worker/males)	**24.0**	16.6	**22.8**	10.2	**14.7**	11.8	**138.0*** a)	46.6
Living pupae (worker/males)	**45.0**	13.1	**101.7**	56.2	**61.0**	50.6	**44.7**	8.1
Dead pupae (worker/males)	**3.5**	3.3	**5.7**	5.1	**10.7**	8.0	**1.3**	1.2
Filled nectar cells	**1.5**	1.8	**4.2**	5.5	**13.8**	7.7	**0.7**	0.6
Filled pollen cells	**2.8**	1.9	**3.8**	2.2	**4.5**	3.0	**7.0**	5.3
Colony weight [g]	**382.8**	67.0	**533.3**	93.9	**496.7**	61.8	**56.0*** b)	6.2
Total living brood stages (eggs, larvae, pupae)	**263.3**	92.5	**431.8**	154.6	**453.5**	142.6	**63.0*** b)	12.1
Total living adult bees (young queens, worker bees, males)	**99.2**	14.0	**153.3**	52.9	**135.0**	17.5	**12.0*** a)	9.8
Total living stages (brood and adult bees)	**362.5**	101.9	**585.2**	176.0	**588.5**	141.3	**75.0*** a)	21.5
STD = standard deviation; - = not available								
Calculation of weights based on unrounded values								
* statistically significant difference compared to control ($p \leq 0.05$)								
a) pooled t-test; b) Satterthwaite t-test; c) Mann Whitney exact								

Drone and queen production

The mean number of emerged young queens and males produced in the control (56.0), T1 (96.3) and T2 (83.7) did not show any statistical differences. Also, no statistical differences in the numbers of living queen brood were observed for T1 with 0.7 queen larvae and 98.7 queen pupae, T2 with 3.5 queen larvae and 117.7 queen pupae compared to 3.8 queen larvae and 85.5 queen pupae in the control. Accordingly, the total number of living queen stages was also similar with 145.3 in C, 195.7 in T1 and 204.8 in T2. The mean queen weight (weighed individually) was not significantly different with 0.89 g in T1 and 0.90 g in T2 compared to 0.87 g in the control (Table 6). The mean number of emerged young males produced in the control (8.5), T1 (11.5) and T2 (9.7) did also not show any statistically significant differences. The numbers of dead queen and male stages were all very low and similar across C, T1 and T2.

Table 6
Mean number of young queens and males (n ± standard deviation (STD)) and mean queen weight (n ± standard deviation (STD)) in chlorantraniliprole treatment group T1 and T2, control (C) and toxic reference (R)

Life stage	**Mean number of young queens and males (adults and brood stages) (n ± standard deviation) and Mean queen weight (g ± standard deviation)**							
	C		**T1**		**T2**		**R**	
	Mean	**STD**	**Mean**	**STD**	**Mean**	**STD**	**Mean**	**STD**
Living males	**8.5**	10.2	**11.5**	6.7	**9.7**	18.4	**0.3**	0.6
Dead males	**0.0**	0.0	**0.5**	0.5	**0.2**	0.4	**0.0**	0.0
Living queen larvae	**3.8**	3.4	**0.7**	1.0	**3.5**	4.1	**0.0*** [a)]	0.0
Dead queen larvae	**0.2**	0.4	**0.3**	0.5	**0.3**	0.5	**0.0**	0.0
Living queen pupae	**85.5**	17.6	**98.7**	50.5	**117.7**	44.8	**0.0*** [a)]	0.0
Dead queen pupae	**16.2**	37.7	**4.7**	4.8	**9.7**	7.7	**0.0**	0.0
Emerged young queens	**56.0**	19.3	**96.3**	38.5	**83.7**	15.2	**0.0*** [a)]	0.0
Dead young queens	**0.0**	0.0	**0.3**	0.5	**0.2**	0.4	**0.0**	0.0
Mean queen weight [g]	**0.87**	0.08	**0.89**	0.04	**0.90**	0.02	**-**	-
Living queen stages (young queens, larvae, pupae)	**145.3**	29.2	**195.7**	55.7	**204.8**	53.1	**0.0*** [b)]	0.0
STD = standard deviation; - = not available								
Calculation of weights based on unrounded values								
* statistically significant difference compared to control ($p \leq 0.05$)								
[a)] Satterthwaite t-test; [b)] Mann Whitney exact								

Residues

In the 20 cm topsoil that was initially sprayed at 265.15 g chlorantraniliprole/ha, 75% of the target rate was found assuming a standard soil bulk density of 1.5 g/cm³ (Table 1). The analytical dose verification of the different Chlorantraniliprole 20SC spray solutions resulted in 93 to 108% recovery of the target rates (Table 1).

No chlorantraniliprole residues above the LOQ of 0.0005 mg/kg were detected in any control samples in pollen and nectar collected from forager bees and from samples taken inside the colonies that were taken in parallel to the samples in T1 and T2. The results of the residue analysis for T1 and T2 are given in Table 7. The chlorantraniliprole residues determined in pollen taken at 1DBA4 were similar in T1 and T2 with residue levels ranging between 0.1350 and 0.3660 mg/kg. In T1 the pollen residue levels showed a clear decreasing trend and at the last sampling date (29DAA4) the pollen residue levels with 0.0013 mg/kg were only slightly above the LOQ. Following the spray application (A4) during *P. tanacetifolia* flowering in T2 the pollen residues increased to 29.7 and 6.75 mg/kg in forager bee pollen and in pollen sampled from wax pots inside the colonies, respectively. During the next 2 to 3 days the residue levels determined in forager bee pollen and in

pollen sampled from wax pots inside the colonies decreased rapidly to roughly 10 to 100-fold lower residue levels. During the following days the pollen residue levels decreased further and at the last sampling date (29DAA4) an in-hive pollen residue level 0.0032 mg/kg was measured. The chlorantraniliprole residue levels determined in nectar were generally much lower than those found in pollen. At the first nectar sampling on 1DBA4 similar residue levels of 0.0030 and 0023 mg/kg were found in T1 and T2. In T1 the nectar residue levels showed a clear decreasing trend and on the last day of exposure in the tunnels (16DAA4) the nectar residue levels with 0.0008 mg/kg were only slightly above the LOQ. After the spray application (A4) during *P. tanacetifolia* flowering in T2 the nectar residues increased to 0.140 and 0.143 mg/kg in forager bee nectar and in nectar sampled from wax pots inside the hives, respectively. During the following days the residue levels determined in both nectar matrices decreased rapidly and at the last sampling date (29DAA4) the in-hive nectar residue level was below LOQ.

Table 7
Residue concentrations of chlorantraniliprole (mg/kg) detected in pollen and nectar from forager bees (Forager) or collected from wax pots inside the hives (Hive) in chlorantraniliprole treatment group T1 and T2, control (C) and toxic reference (R)

	Pollen (mg chlorantraniliprole/kg)				**Nectar (mg chlorantraniliprole/kg)**			
	T1		**T2**		**T1**		**T2**	
	Forager	**Hive**	**Forager**	**Hive**	**Forager**	**Hive**	**Forager**	**Hive**
1DBA4	0.1720	0.3440*	0.1350	0.3660	<LOQ*	0.0030	0.0023	-
0DAA4	0.1900	0.3340	29.7000	6.7500	0.0031	0.0032	0.1400	0.1430
1DAA4	0.2110	0.2320	3.8000	2.3800	0.0029	0.0021	0.0099	0.0075
2DAA4	0.0735	0.1020	0.4660	0.3520	0.0021	0.0027	0.0112	0.0113
3DAA4	0.0557	0.0433	0.2100	0.5440	0.0020	0.0026	0.0037	0.0043
4DAA4	0.0255	0.0412	0.1180	0.2240	0.0022	<LOQ	0.0039	0.0067
5DAA4	0.0614	0.2580	-	0.0116	0.0017	<LOQ	0.0066	0.0079
6DAA4	0.0247	0.0192	0.1210	0.1660	0.0008	0.0009	0.0030	0.0050
7DAA4	0.0112	0.0156	0.0855	0.1310	0.0012	0.0014	0.0027	0.0041
10DAA4	0.0117	0.0071	0.0268	0.0181	0.0010	<LOQ	0.0022	0.0048
14DAA4	0.0335	0.0262	0.0135	0.0092	0.0015	n.d.	0.0015	0.0015
16DAA4	0.0369	0.0191	0.0185	0.0275	0.0008	0.0008	0.0013	0.0021
22DAA4	-	0.0039	-	0.1110	-	-	-	0.0010
29DAA4	-	0.0013	-	0.0032	-	-	-	<LOQ
* Sample taken 2DBA4								
n. d. = not detectable								
- = the collected amount was not sufficient for the analytical procedure/no samples of forager bees taken after 16 DAA4								
LOQ (Limit of quantification): 0.0005 mg chlorantraniliprole/kg								

Discussion

In this bumblebee tunnel study, young queen-right colonies with initially about 50 worker bumblebees per colony were exposed to untreated and treated flowering *P. tanacetifolia* over a 20-day period and then kept at a monitoring site until approx. 30–40 % of the estimated queen pupae had emerged. The control colonies developed similarly, and young queen and male stages were found in all control colonies at the final colony assessment demonstrating that it is possible to generate consistent and good quality data following the ring-test protocols from the ICPPR Non-Apis workshops (2016 and 2017).

The experiment was conducted under OECD GLP ensuring proper study conduct and data documentation. The analytical dose verification of sprayed (and mixed) soil as well as of spray solutions applied pre and during flowering of *P. tanacetifolia* confirmed the intended chlorantraniliprole concentrations and worst-case exposure scenarios. Concurrently the lack of chlorantraniliprole residues found in the soil, pollen and nectar control samples (all < LOQ of 0.0005 mg/kg) document the lack of exposure of the control bumblebee colonies to chlorantraniliprole. In the toxic reference group, a clear impact on mortality, foraging activity and colony development including young queen and male production was found demonstrating the sensitivity of the semi-field tunnel test system to detect potential effects of plant protection products on bumblebee colonies.

To simulate a worst-case scenario for the intended uses of Chlorantraniliprole 20SC, the product was sprayed at 265.15 g chlorantraniliprole/ha onto bare soil and incorporated into the 20 cm top soil layer in which the *P. tanacetifolia* crop was grown and then received two further spray applications with Chlorantraniliprole 20SC either twice during pre-flowering (T1) or during pre- and flowering period (T2) each at 60 g chlorantraniliprole/ha. The findings of this study indicate no negative effects on *B. terrestris* bumblebee colonies in the chlorantraniliprole treatment T1 and T2 compared to the control with regard to all parameters assessed during the study, i.e. mortality of adult and larval stages, flight activity at the hive entrance, colony weight development, condition of the colonies and production of young queens and males. Only flight activity at the hive entrance was lower one time in T2 (0DAA4) and observed to be higher one time in T1 (5DAA4). But flight activity values are generally more variable compared to other endpoints. Thus, it is not unusual to find differences on single days between the treatment groups. No differences in the mean flight activity during the other days and during the whole exposure period inside the tunnels were found between C, T1 and T2.

The lack of effects found in this study are in line with results determined in an earlier bumblebee semi-field study with queen-right *B. terrestris* colonies that were exposed to two spray applications conducted each at 60 g chlorantraniliprole/ha during *P. tanacetifolia* flowering (Dinter and Brugger 2015). Also, lack of effects on foraging activity, adult mortality, colony weight and queen production was determined for the bumblebee, *B. impatiens*, foraging on flowering white clover that was treated at 230 g chlorantraniliprole/ha, while for another tested insecticide (clothianidin) effects were found (Larson et al. 2013). For *Bombus impatiens* a laboratory study concluded that insect control formulations containing chlorantraniliprole are safe for greenhouse use in the presence of bumblebees (Gradish et al. 2010). Likewise, no toxicity of chlorantraniliprole to *B. terrestris* colonies sprayed at 40 g a.s./ha during foraging activity in tomato in a Spanish greenhouse was determined (Dinter et al. 2009).

Only a worst-case laboratory chronic 7-week oral exposure study with small artificial *B. terrestris* colonies without a queen (and instead a pseudo-worker queen) and with constant exposure to chlorantraniliprole via sugar water at 4 to 40 mg a.s./L or pollen dosed at 0.4 to 40 mg a.s./kg found suppression of reproduction in form of reduced drone production (Smagghe et al. 2013). When comparing the actual dose rates that Smagghe et al. 2013 tested with the measured chlorantraniliprole concentration found in this worst-case semi-field tunnel trial, it becomes obvious how unrealistically high and continuously the laboratory study reported in Smagghe et al. 2013 was dosed. The maximum chlorantraniliprole concentrations measured in the current semi-field study were 0.143 mg a.s./kg for nectar and 29.7 mg a.s./kg for pollen. These residues levels were detected in the treatment T2 samples that had received Chlorantraniliprole 20SC spraying during flowering of *P. tanacetifolia* and were taken a few hours after spray application. The residue levels determined in forager bee pollen and in pollen sampled from wax pots inside the colonies decreased rapidly to roughly 10 to 100-fold

lower residue levels during the next 2 to 3 days. Also, the nectar residue levels decreased rapidly in T2 during the following days after the spray application made during flowering of *P. tanacetifolia*. Thus, the chlorantraniliprole pollen concentrations measured under worst-case semi-field conditions in T2 are only for a very few days overlapping with the concentrations tested over 7 weeks by Smagghe et al. (2013). Similarly, all measured chlorantraniliprole active ingredient nectar concentrations in the current tunnel study were lower than the concentrations causing reproductive effects reported by Smagghe et al. (2013).

Overall, the pollen and nectar residue data of the current tunnel study demonstrate that such continuous high-dose laboratory exposure scenarios – as investigated by Smagghe et al. (2013) – are unrealistic and over-conservative for bumblebee exposure to chlorantraniliprole for the intended and registered uses of chlorantraniliprole containing products and assuming a modelled worst-case 20-year plateau concentration of chlorantraniliprole in the top 20 cm of soil plus one pre-flowering spray application and one spray application made during bumblebee flight during the day at 60 g a.s./ha. At the same time the pollen and nectar residue results demonstrate that the chlorantraniliprole pollen and nectar residue levels are significantly lower (about 2 orders of magnitude) from pre-flowering spraying versus spraying during flowering and bee flight. Beyond, the pollen and nectar residue results highlight that there is no obvious difference in the residue level if the *P. tanacetifolia* crop had received one (in case of T2) or two (in case of T1) Chlorantraniliprole 20SC sprays as the residue levels detected on 1DBA4 were similar in T1 and T2.

Conclusions

When Chlorantraniliprole 20SC was applied once to the soil followed by soil incorporation before *P. tanacetifolia* seeding at a modelled worst-case 20-year plateau concentration and then applied twice as foliar spray on pre-flowering or flowering *P. tanacetifolia*, all parameters assessed (mortality, flight activity, colony weight, condition of the colonies and production of young queens and males) did not have any treatment-related effects compared to the water-treated control. Also, there was no difference between the two chlorantraniliprole treatment scenarios T1 (pre-flowering exposure) and T2 (pre-flowering plus spray during flowering and during bee flight). Overall, no effects of chlorantraniliprole on bumblebee *B. terrestris* colonies including queen and drone production and adult and larval mortality were found.

Declarations

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Compliance with ethical standards

Conflict of interest

The authors declare that they have no competing interests. Funding of all expenses for this study was provided by FMC Agricultural Solutions. All practical work, data and statistical analysis were done by Eurofins Agroscience Services Ecotox GmbH.

Ethical approval

All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

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Figures

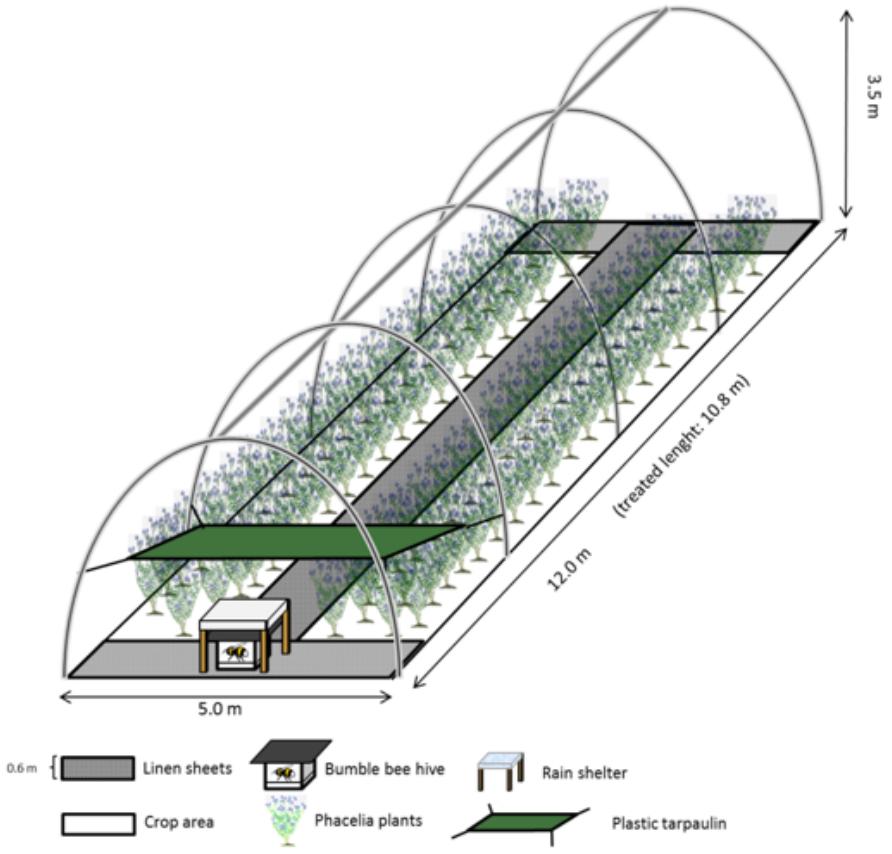

Figure 1

Setup of the tunnels and bumblebee colonies and the linen sheets in the tunnels for biological assessments (C, T1, T2, R)