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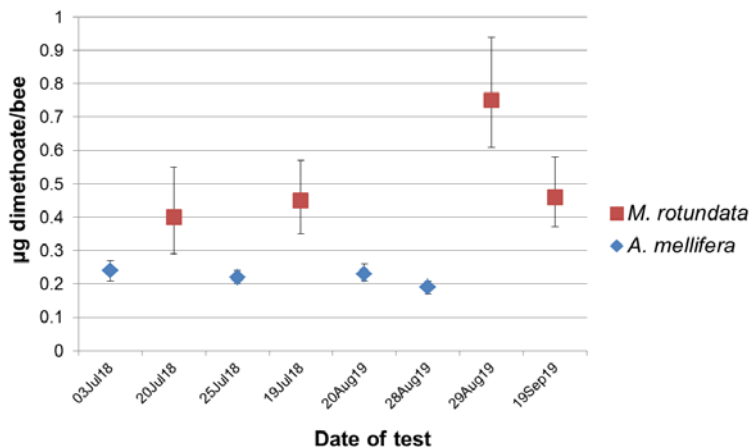


Fig. 2 LD₅₀/24h values (error bars indicate 95% confidence levels) of *M. rotundata* and *A. mellifera* determined at about the same time period 2018 and 2019

Conclusions

The mortality did not exceed 20% in all control treatments (water or acetone) with *M. rotundata* after 72 hours. The low mortality observed in the control also after 96 hours, confirms the feasibility and reliability of the test method.

The LD₅₀/24h values for formulated dimethoate in both bee species were reproducible (*A. mellifera*: 0.19 – 0.24 µg dimethoate/bee; *M. rotundata*: 0.40 – 0.75 µg dimethoate/bee) and could be determined with good precision according to the classification provided by EFSA (2019).

The LD₅₀/24h values of *M. rotundata* in all for tests were higher compared to those of *A. mellifera*. Accordingly, *M. rotundata* appeared to be slightly less sensitive to formulated dimethoate than *A. mellifera*.

Pure acetone was tolerated by *M. rotundata* and did not cause higher mortality compared to water treatment. Hence, acetone is a solvent which can be used in acute contact toxicity tests with *M. rotundata*.

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- EUROPEAN FOOD SAFETY AUTHORITY, ARENA, M., AUTERI, D., BARMAZ, S., CHAIDEFTOU, E., CTVERACKOVA, L., ... & MOLNAR, T. (2019). Outcome of the Pesticides Peer Review Meeting on general recurring issues in ecotoxicology. EFSA Supporting Publications, 16(7), 1673E.
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4.4.P Recent experiences with bumblebee (*Bombus terrestris*) semi-field tunnel testing following ICPPR Non-Apis 2016 and 2017 workshop recommendations to investigate the insecticide chlorantraniliprole

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Abstracts: Poster**Abstract**

The study investigated the potential impact of the insecticide chlorantraniliprole (Coragen® brand) on the bumble bee (*Bombus terrestris* L.) under semi-field conditions in *Phacelia tanacetifolia* in Germany based on ringtest protocols from the ICPPR Non-Apis workshops (2016 and 2017). The *P. tanacetifolia* crop was grown in soil treated with the predicted 20-year plateau concentration of chlorantraniliprole in the top 20 cm of soil (equivalent to a predicted 20-year plateau concentration of 0.088 mg a.s./kg). Additionally, two chlorantraniliprole applications at 60 g a.s./ha were made in the chlorantraniliprole treatments (T1 and T2). In T1 both applications took place before *P. tanacetifolia* flowering at BBCH 51-55 and BBCH 55-59. In T2 one 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 bumble bee flight. The bumble bee 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. The results of this study indicate no significant differences between the chlorantraniliprole treatment groups T1 and T2 and the control regarding all parameters assessed (i.e. mortality in the colonies and in the tunnels, flight activity at the hive entrance, hive weight development, condition of the colonies and production of young queens and males). Overall, no effects of chlorantraniliprole on bumble bee *B. terrestris* colonies including queen/male production, adult and larval survival and forager flight activity were found.

Keywords: Bumble bee, *Bombus terrestris*, chlorantraniliprole, insecticide

Introduction

The objective of the study was to determine the effects of the insecticide chlorantraniliprole 20SC (Coragen® brand, 200 g chlorantraniliprole active substance/L) on the bumble bee (*Bombus terrestris* L.) in two test item treatment groups T1 and T2 under semi-field conditions in *Phacelia tanacetifolia* in Germany based on general SETAC/ESCORT recommendations (BARRETT et al. 1994), EPPO Guideline No. 170 (4) (2010) and ringtest protocols from the ICPPR Non-Apis workshops (2016 and 2017). Chlorantraniliprole, an anthranilic diamide insecticide with a favorable profile for numerous beneficial arthropods (Dinter et al 2008), was investigated to assess the potential for effects on the bumble bee in field use conditions. *P. tanacetifolia* was used as a high pollen and nectar-producing, bee-attractive crop. Chlorantraniliprole was 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 either during pre-flowering or during pre- and flowering period.

Materials and Methods

The study was located outside Pforzheim in Southern Germany and conducted in 2019. Six replicate tunnels were set up each for the water-treated control (C) and the chlorantraniliprole treated groups T1 and T2, and three replicate tunnels for the toxic reference (R) each with a tunnel size of approx. 60 m² and one bumble bee colony per tunnel.

The first application (A1) of the test item chlorantraniliprole 20SC was applied to bare soil in mid-April 2019 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 predicted 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:

T1 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).

T2 applications were conducted once before *P. tanacetifolia* flowering (BBCH 55-59 (A3)) and once during *P. tanacetifolia* flowering and during daily bee flight (BBCH 61-62 (A4)) with an 8-day spray interval.

The application in the control (C) (water only) and reference item treatment (R) (400 g dimethoate a.s./ha) was carried out during full *P. tanacetifolia* flowering and bumble bee flight on the same day as the 2nd application of T2 (A4). All spray applications were performed with a water volume of 300 L tap water/ha.

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Young commercial queen right colonies (origin Koppert BV) with 45 to 69 worker bumble bees per colony were set up inside the tunnels after the initial brood assessment at BBCH 59-61 on 14 June 2019 three days before application A4 (= 3DBA4). The bumble bee colonies were exposed to the treated flowering *P. tanacetifolia* crop for 20 days in the tunnels and did not receive any supplementary feeding with sugar solution during the experiment as is typically provided with commercial bumble bee colonies that may be used in crops which do not provide nectar (e.g. tomatoes) (worst-case scenario). 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 bumble bee brood. At the end of flowering of *P. tanacetifolia* (BBCH 69) the bumble bee hives were transferred to a 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. Then each hive was individually deep-frozen. When it was foreseeable that a colony would not reach the switch-point to produce reproductives, it was deep-frozen earlier (replicate Ra, Rb and Rc). At the end of the study after deep-freezing of all colonies, a final brood assessment was conducted to get a detailed overview of the colony brood development. The statistical software program SAS Version 9.4 was used for the statistical analysis.

Results

The influence of the test item chlorantraniliprole 20SC and the toxic reference item dimethoate were evaluated by comparing the results of the test item and the toxic reference item treatments to the data in the control treatment regarding the following observations: Mortality of adult worker bumble bees in tunnels and in hives, flight activity, mortality of larvae in hives, development of brood nest (weight of hive), development of bumble bee brood (brood assessment), and young queens and males production.

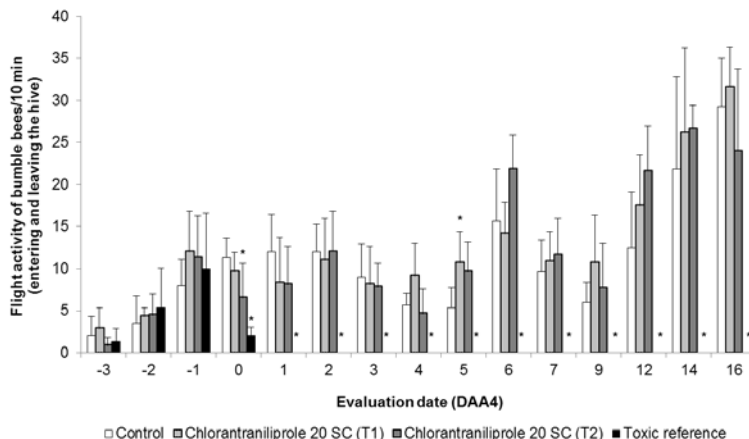
Bumble bee flight intensity

The bumble bee hives were placed in all tunnels 3 days before application A4 when first flowers were open to acclimatize the bumble bees in the tunnels until the application. In all treatment groups the bumble bees started to forage immediately after the set-up on -3DAA4 with 0.8 to 2.8 entering and leaving bumble bees per 10 min (Fig. 1). In 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 bumble bees at the hive entrance in 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 bumble bees/10 min, but flight activity was observed to be slightly but significantly lower in T2 with 6.5 bumble bees at the hive entrance in 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). There were no statistically significant differences in mean flight activity in C, T1 and T2 during the whole exposure period with 10.9, 12.5 and 11.9 entering and exiting bumble bees/10 min, respectively. The flight activity in the toxic

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reference 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).

Fig. 1 Mean flight activity (number of forager bees/ 10 min \pm STD) of bumble bees at the hive entrance of the control, the chlorantraniliprole groups T1 and T2 and the toxic reference during tunnel exposure phase. (DAA4 = days after 4th application. * statistically significant difference to control ($p \leq 0.05$, Dunnett's t-test, pooled t-test, Satterthwaite t-test, Mann Whitney exact test)).



Bumble bee adult worker mortality inside the hives

Before the application A4 from -3DAA4 to -1DAA4 the mortality was low and not statistically significant different in the control, the chlorantraniliprole groups T1 and T2 and the toxic reference with maximum values of 0.3 dead workers per day (Fig. 2). Mortality of workers 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 inside the colonies, respectively. In the toxic reference mortality of adult bumble bees was statistically significantly higher compared to the control on all assessment days from 0DAA4 to 16DAA4 ($p \leq 0.05$, 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 during the exposure period was also statistically significantly higher with 94.3 dead workers in the toxic reference compared to 2.5 in the control ($p \leq 0.05$, Mann Whitney exact test).

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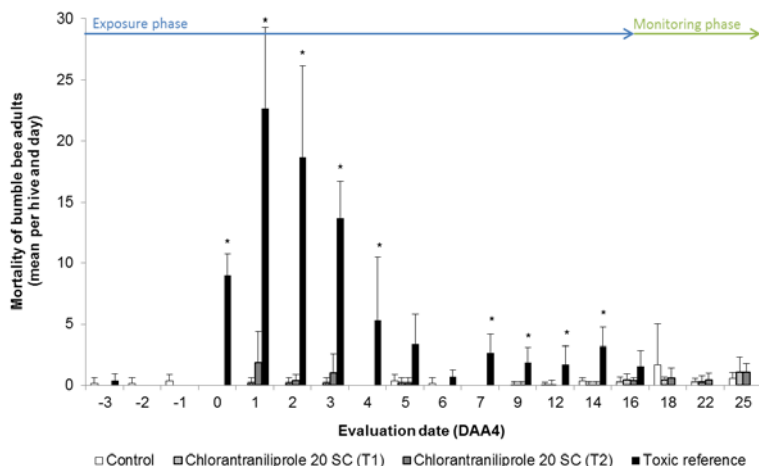


Fig. 2 Number of dead bumble bees per day (adult worker) collected inside the colonies of the chlorantraniliprole groups T1 and T2 and the toxic reference (DAA4 = days after 4th application. * statistically significant difference to control ($p \leq 0.05$, Satterthwaite t-test, Mann Whitney exact test)).

Bumble bee queen mortality inside the hives

Foundress queen mortality was observed on 2DAA4 in replicate T2c. The reason for the mortality of the queen was not clear, but it could be natural background mortality. The foundress queen was replaced with a foundress queen from a similarly treated hive (from a separately similarly treated reserve tunnel). Except for replicate T2c no foundress queen mortality was observed in any of the control or test item treatment T1 and T2 colonies. In the toxic reference all foundress queens died within the first 5 days after the application. First queen pupae in the control and T1 and T2 were observed between 9DAA4 and 16DAA4 and first young queens emerged between 22DAA4 and 32DAA4. No queen brood was observed in the colonies of the toxic reference. As none or only few workers (0 to 4) were still alive in the reference colonies, it was foreseeable that these colonies would not reach the switch-point, so the colonies were deep-frozen on the day of transfer to the monitoring site (17DAA4).

Bumble bee larvae mortality inside the hives

From -3DAA4 to -1DAA4 the larval mortality was low with maximum values of 0.3 dead larvae per day and not statistically significant different in the control, T1 and T2 and the toxic reference. After the application A4 the mortality of larvae in the hives stayed at a low level with maximum values of 0.7 dead larvae per day in C on 5DAA4, 1.3 in T1 on 1DAA4 and 1.2 in T2 on 3DAA4. No statistically significant differences were observed in T1 and T2 compared to the control during the study from -3DAA4 to 22DAA4. On 25DAA4 an increase in larval mortality was seen in the control and T1 and T2 due to the natural senescence of the colonies, as the worker numbers declined in all colonies at the end of the monitoring phase and thus the provisioning of the larvae decreased. No statistically significant differences in mean total mortality during the exposure phase were found with 4.2, 5.8 and 4.5 dead larvae inside the colonies in C, T1 and T2, respectively. In the toxic reference a statistically significant higher larval mortality was observed on 1DAA4 and 6DAA4 ($p \leq 0.05$, Mann Whitney exact test).

Bumble bee adult and larvae mortality collected inside the tunnels on linen sheets and in front of the hives

Mortality values of bumble bee larvae and adults determined on linen sheets and in front of the hives were very low and not statistically significant different throughout the study, with maximum

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values of 0.3, 0.3 and 0.2 dead larvae and workers in the tunnels per day in the control, T1 and T2, respectively. No statistically significant differences in mean total mortality during the exposure phase were found in T1 and T2 with 0.5 and 0.2 dead bumble bees found in the tunnels compared to 0.3 in the control. In the toxic reference mortality in the tunnels was also low, with maximum values of 1.7 in the toxic reference compared to 0.3 in the control. No statistically significant differences were found on any assessment day. However, the mean total mortality showed a statistically significant increase with 3.0 dead bumble bees in the tunnels of the toxic reference compared to 0.3 in the control ($p \leq 0.05$, Mann Whitney exact test).

Bumble bee colony weight

The mean colony weight values are presented in Fig. 3 from -3DAA4 until the first bumble bee colonies were deep-frozen in the control and the test item treatment groups. No statistically significant differences in the colony weight development were detected between the control and the T1 and T2 throughout the study. After the colonies were acclimated in the tunnels, 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 gain 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 from -3DAA4 until 1DAA4 and decreased from 2DAA4 onwards. The mean total weight gain from -3DAA4 until deep-freezing of the colonies was statistically significantly lower in the toxic reference compared to the control ($p \leq 0.05$, Satterthwaite t-test).

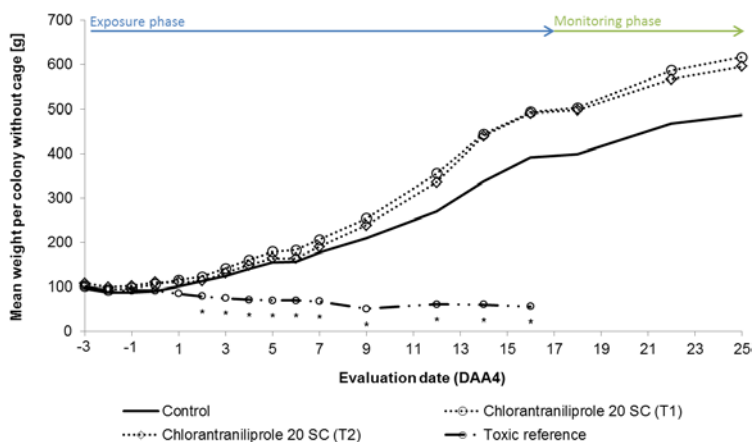


Fig. 3 Mean weight (g) of the colonies of the control, chlorantranilprole groups T1 and T2 and the toxic reference during tunnel exposure phase and monitoring phase. (DAA4 = days after 4th application; The net colony weight is presented in the figure (without the weight of the plastic cage). * statistically significant difference to control ($p \leq 0.05$, pooled t-test, Satterthwaite t-test)).

Bumble bee colony and brood assessments

At the initial brood assessment before the bumble bee colonies were set up in the tunnels, all bumble bee colonies chosen for the control, T1 and T2 and the toxic reference 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, the hives of the control, the T1 and T2 and the toxic reference showed similar strength with regard to the number of living brood stages and food storage. No statistically significant differences in the condition of the bumble bee colonies of T1 and T2 and the toxic reference compared to the control were observed at the initial brood assessment.

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At the end of the monitoring phase the bumble bee hives were deep-frozen individually when approx. 30% 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 hives in the toxic reference did not reach the switch-point before deep-freezing. They were deep-frozen as soon as it was foreseeable that they would not reach the switch-point and would not produce any queens. After deep-freezing the final brood assessment was conducted. 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 found to be statistically significantly 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.

The similarity of the bumble bee colonies is also clear on basis of photographic documentation and photographs taken at the final brood assessment. Exemplary a bumble bee colony picture (without adult bees) is given for control, T1 and T2 taken at the final brood assessment (Fig. 4).

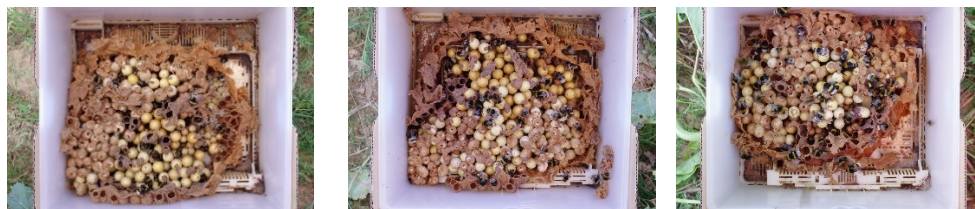


Fig. 4 Pictures of a control, chlorantraniliprole groups T1 and T2 colony at the final colony assessment (from left to right).

Young queen and male production

Fig. 5 shows the mean number of emerged young queens and males collected in the colonies during the monitoring phase and during the final brood assessment. The mean number of emerged young queens and males produced in the control (56.0 and 8.5), T1 (96.3 and 11.5) and T2 (83.7 and 9.7) did not show any statistically significant differences. There were no statistically significant differences in the number of queen brood 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 204.5 in T2. 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.

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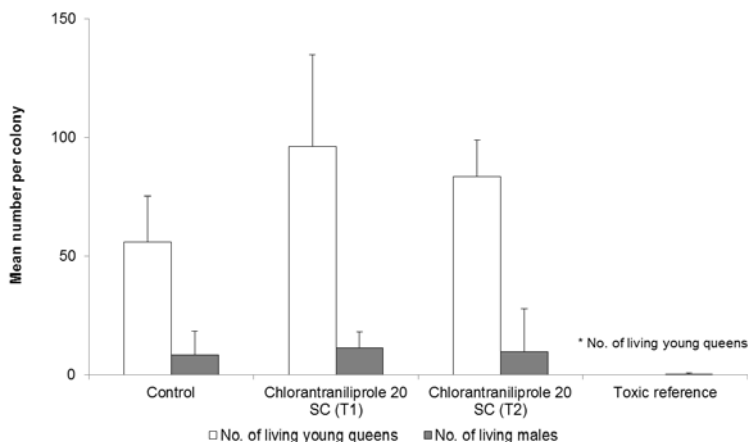


Fig. 5 Mean number of living young queens and males of the colonies of the of the control, chlorantraniliprole groups T1 and T2 and the toxic reference at the final colony assessment. (* statistically significant reduction compared to control ($p \leq 0.05$, Satterthwaite t-test).

Discussion

In the current bumble bee tunnel study it was possible to expose young commercial queen-right colonies with initially about 50 worker bumble bees per colony to untreated and treated *P. tanacetifolia* over the whole 20-day flowering period. Afterwards the colonies were kept at a monitoring site until approx. 30-40 % of the estimated queen pupae had emerged. The colonies of the control group developed similarly, and young queen and male stages were found in all control colonies at the final colony assessment. The study demonstrates that it is possible to generate consistent and good quality data following the ringtest protocols from the ICPPR Non-Apis workshops (2016 and 2017) while efforts of other research groups were often not successful generating data on reproductive performance.

The results of this study indicate no treatment-related impacts on bumble bee colonies between the chlorantraniliprole treatment groups T1 and T2 and the control with regard to the parameters assessed during the study, i.e. mortality in the colonies and in the tunnels, flight activity at the hive entrance, hive weight development, condition of the colonies and production of young queens and males were determined with the following exceptions: Flight activity at the hive entrance was lower one time in T2 (0DAA4) and observed to be higher one time in T1 (5DAA4). Generally, flight activity values are 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 whole exposure period were found between C, T1 and T2.

A worst-case laboratory chronic oral exposure study with small artificial *B. 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 bumble bees (Smagge et al 2013). But such continuous high-dose laboratory exposure scenarios for bumble bees to chlorantraniliprole are unrealistic and highly conservative. In an earlier bumble bee semi-field study with *B. terrestris* colonies also on negative impact on reproduction of bumble bees was found (Dinter & Brugger 2015). Lack of effects on foraging activity, adult mortality, colony weight and queen production were also found for the bumble bee, *B. impatiens*, foraging on flowering white clover that was treated with 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 chlorantraniliprole is safe for greenhouse use in the presence of bumble bees (Gradish et al 2011). Low toxicity and low risk for honey bees and *B. terrestris* was demonstrated for chlorantraniliprole

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and its formulated products in several worst-case semi-field tunnel and greenhouse trials (Dinter et al 2009).

Conclusions

When chlorantraniliprole was applied once to the soil followed by soil incorporation before *P. tanacetifolia* seeding at a predicted 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 bumble bee *B. terrestris* colonies including queen production and adult and larval mortality were found.

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4.5.P Sensitivity of the honey bee and different wild bee species to plant protection products – two years of comparative laboratory studies

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Abstract

Effects of active substances have been tested mainly on honey bees and occasionally on a few other commercially used bee species with regard to registration processes and risk assessment of plant protection products (PPPs). However, toxicity data are lacking for the majority of wild bee species. The aim of these