Performance of honey bee colonies under a long-lasting dietary exposure to sub-lethal concentrations of the neonicotinoid insecticide thiacloprid[1].

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Abstract

BACKGROUND

Substantial honey bee colony losses have occurred periodically in the last decades. The drivers for these losses are not fully understood. The influence of pests and pathogens are beyond dispute, but in addition, chronic exposure to sub-lethal concentrations of pesticides has been suggested to affect the performance of honey bee colonies. This study aims to elucidate the potential effects of a chronic exposure to sub-lethal

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concentrations (one realistic worst-case concentration) of the neonicotinoid thiacloprid to honey bee colonies in a three-year replicated colony feeding study.

RESULTS

Thiacloprid did not significantly affect the colony strength. No differences between treatment and control were observed for the mortality of bees, the infestation with the parasitic mite *Varroa destructor* and the infection levels of viruses. No colony losses occurred during the overwintering seasons. Furthermore, thiacloprid did not influence the constitutive expression of the immunity related hymenoptaecin gene. However, upregulating of the hymenoptaecin expression as a response to bacterial challenge was less pronounced in exposed bees than in control bees.

CONCLUSION

Under field conditions bee colonies are not adversely affected by a long-lasting exposure to sub-lethal concentrations of thiacloprid. No indications were found that field-realistic and higher doses exerted a biologically significant effect on colony performance.

Key words

Honey bee colony, performance, thiacloprid, neonicotinoids, chronic exposure

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1 INTRODUCTION

Thiacloprid is an insecticide belonging to the chemical class of the neonicotinoids. These substances show structural similarities with nicotine and first came to the market in 1991, with imidacloprid as the first substance to be commercialized. Since further neonicotinoids, such as acetamiprid, clothianidin, thiamethoxam and thiacloprid have realized high shares on the insecticide markets. In 2014 around 20% of all domestically traded insecticides in Germany belonged to the nicotinoids group. [1] Of the insecticides exported from Germany they account for over 50 % of the market.[1] Thiacloprid is used as active substance in products for foliar applications to control pests in orchards, arable crops, vegetables production and other specialized crops, and as a seed treatment in maize. In various countries in Northern, Western and Central Europe an important field of application for products containing thiacloprid is the foliar treatment of oilseed rape (*Brassica napus*) at early flowering. Treatments during full blossom of the crop are permitted in some countries as thiacloprid is characterized by a low to moderate toxicity for bees, and application during bloom are consequently classified as safe to bees.[2] Honey bees can relatively quickly detoxify neonicotinoids such as thiacloprid which contain a cyano-substitution in their imidazolidine ring.[3] In contrast, nitro-substituted neonicotinioids, such as imidacloprid, are not as quickly metabolized by the detoxification system of the bees, and therefore show a substantially higher intrinsic toxicity to individual honey bees (e.g. imidacloprid: 3.7 – <104 ng bee^{-1}; LD_{50} 48h after oral uptake). The intrinsic toxicity of thiacloprid to bees is lower by the factor of ca. 1000 (17.3µg bee^{-1} LD_{50} 48h after oral uptake).[4] However, due to the low to moderate toxicity of thiacloprid, the substance was frequently applied into flowering crops, meaning that foragers collecting nectar and pollen from treated crops may carry substantial amounts of thiacloprid residues back to their hives. Indeed, thiacloprid

residues have been found in stored honey and in bee bread containing oilseed rape pollen. [5-7] Consequently, colonies might be exposed to sub-lethal concentrations of thiacloprid over long periods. Several studies investigated the effects of both toxic and sub-lethal concentrations of thiacloprid on individual honey bees, demonstrating that thiacloprid can affect neuronal functions as well as orientation and homing behavior,[8] and survival under starvation stress [9] and pathological stress.[10, 11] However, the relevance of long-lasting exposure of thiacloprid to colonies has so far not been well understood. In this study, we evaluated possible effects of thiacloprid on free ranging colonies under field-realistic conditions in a replicated design over three consecutive years. Exposure to the test substance concentrations was achieved by in-hive feeding.

2 MATERIALS AND METHODS

2.1 Study design

Over three years, from July 2011 to May 2014, three groups of ten *Apis mellifera carnica* colonies each were set up for the tests. The experiment was replicated three times between 2011 and 2013. Exposure to thiacloprid was achieved by in-hive feeding of spiked sugar syrup which was administered in plastic hive top feeders. Control colonies ('C') were provided with syrup containing the solvent acetone without thiacloprid. Treatment groups received syrup containing either 0.2 mg thiacloprid L^{-1} ('T1') or 2 mg thiacloprid L^{-1} ('T2'). The colonies were set up in an apiary situated in an agricultural area close to Kirchhain, Germany. During the exposure period, no major bee-attractive crops were flowering within a 3 km flight radius of the hives. Colonies of each treatment group were placed in rows. Each block of hives was separated from the other treatment groups by a distance of approx. 20 m. The entrances of the hives were

oriented towards the east. Each year new colonies with thoroughly cleaned hive equipment were used to ensure optimized standardization. Heat stable material as wooden hive boxes, bottom boards, supers and queen excluders were scraped and cleaned with hot water high pressure cleaners. Plastic parts were cleaned with cold NaOH lye. Frames were boiled in hot NaOH lye and filled with wax-foundations. All material was color-coded and only re-used in the corresponding treatment groups. The placement of the blocks of the treatment groups rotated over the three years.

2.2 Colony management

Each year at the beginning of July 30 shook swarms were obtained from a commercial beekeeper. The shook swarms were weighed, equilibrated to 2 kg bees, queened with 4-week-old-sister-queens and treated against *Varroa destructor* with coumaphos (Perizin ®). Few weeks before the beginning of the experiment all queens had been mated at the mating station of Gehlberg (Germany). The swarms were kept in a cool room for 48 h and then were moved to the apiary. The colonies were set up in hives with 8 frames of wax foundation and two empty drawn combs, and were immediately fed with 5 L sugar syrup. After 7 days a further 5 L of syrup were provided, and thereafter feeding continued with aliquots of a 5 L syrup at intervals of 21 days. At the end of the feeding period, each colony had been provided with a total of 25 L syrup which was enough food to carry them through the winter. In December, the coumaphos treatment against *Varroa* was repeated. In spring, an additional brood chamber, a honey super and one or two drone combs were added according to colony development. In cases of queen losses, sister queens of a reserve stock were added to the affected colonies.

2.3 Exposure to thiacloprid

Two nominal thiacloprid concentrations were administered to the colonies of the respective treatment groups, T1 with 0.2 mg thiacloprid L^{-1}, and T2 with 2 mg thiacloprid L^{-1}. Dosing was derived from realistic field exposure data: according to the findings of the German Bee Monitoring,[6] Thiacloprid residue levels of ca. 0.2 mg kg^{-1} were high-end concentrations in bee bread samples collected from bee hives in Germany, therefore representing a realistic worst-case scenario, and 2 mg kg^{-1} were a ten-fold increased concentration in order to depict an unrealistic worst-case scenario. Thiacloprid (content of active ingredient 98.3% w/w certified by Bayer AG laboratory) was obtained from Bayer AG. Each year, stock solutions of 2 mg thiacloprid 1 ml^{-1} acetone and 20 mg thiacloprid 1 ml^{-1} acetone were prepared in glass flasks and stored in a dark and cool room (~ 15°C) until use. Several days before feeding 5.4 ml of each stock solution, or 5.4 ml of pure acetone were added to 54 L sugar syrup (Ambrosia ®; 0.73 kg sugar L^{-1}) to obtain syrup for controls without thiacloprid, syrup containing 0.2 mg thiacloprid L^{-1} (≈ 0.139 mg kg^{-1}) for group T1 and syrup containing 2 mg thiacloprid L^{-1} (≈ 1.39 mg kg^{-1}) for group T2. The mixtures were thoroughly stirred for 30 min with a whisk at high speed. From each mixture, samples were retained for chemical analysis. Samples were sent to a commercial laboratory (eurofins, Dr. Specht, Hamburg, Germany), for thiacloprid content determinationby the QuEChERS method according to DIN EN 15662:2009. The analytically confirmed concentrations in the feeding syrup were slightly below the targeted concentrations. In syrup for the control group no thiacloprid was detectable (N: number of analyzed syrup samples = 11). In syrup for group T1 a mean of 0.125mg kg^{-1} thiacloprid (≈ 0.178 mg L^{-1} ; Min. 0.04 mg kg^{-1} ; Max. 0.19 mg kg^{-1}, N = 15) was found, syrup for T2 contained 1.005 mg kg^{-1} thiacloprid (≈ 1.437 mg L^{-1} ; Min. 0.110 mg kg^{-1} ; Max. 1.800 mg kg^{-1}, N = 15).

2.4 Pathological characterization of the colonies

Approximately 40 g of living bees were brushed off the outer combs of each colony at the beginning of August (4 weeks after initiation of the experiment = wai), mid of September (10 wai) and beginning of October of each year (13 wai). Samples were weighed. Bees were shaken in soapy water to remove *Varroa* mites and detached mites were retained by filtering the suspension through a honey strainer double sieve. The detached *Varroa* mites were counted.[12] In addition, 60 bees per colony were sampled from the outer combs in July, September and March for *Nosema* spp.. The numbers of spores of *Nosema* spp. were microscopically determined with the help of a hemocytometer.[13] In the middle of September of each year a further ten bees were collected from the outer combs for virus analysis. Nucleic acids (NA) were extracted with the QIAamp Viral RNA Mini Kit according to the instructions of the manufacturer. Extracts were checked photometrically for purity and yield of NAs. Preparations were diluted to 20 ng μl^{-1} NA before use in PCR. One-step qRT-PCRs (QuantiTect SYBR Green RT-PCR, Qiagen, Hilden, Germany) were set up with diagnostic primers for ABPV (oligonucleotides A and B,[14] DWV [15] and CBPV).[16] The housekeeper Rp49 was used as reference gene and amplification control. It was amplified with primers according to de Miranda and Fries (2008).[17] PCR conditions corresponded to common standards. Heavily loaded field extracts were used as positive controls. Pure water instead of RNA extracts were used as negative controls. Amplicons were checked by melting curve analysis and by separation in agarose gels or, alternatively, by automated gel electrophoresis with a QIAxcel device (Qiagen Hilden Germany).

2.5 Determination of immunological parameters

Thirty 7 day old worker bees were collected from each colony at the end of August / beginning of September of each year. To obtain 7 day old worker bees wire cages were fixed on combs with emerging brood. One day later, the freshly emerged bees were collected, marked with a colored dot and released back into their respective colonies. Seven days later, 10 marked bees per colony were collected into metal cages, brought to the laboratory and supplied with either pure sugar syrup (control bees), or syrup containing 0.2 mg thiacloprid L^{-1} for bees from group T1 and syrup containing 2 mg thiacloprid L^{-1} for bees from group T2. The next day, five bees per cage were anaesthetized on ice, injected with 3 µl of a suspension containing spores and cells of *Paenibacillus larvae* (OD_{600}= 1.4), and transferred to a new cage. One day later, three of the injected and three of the non-injected bees were shock frozen on dry ice and stored at -80°C until extraction of RNA. From each individual bee RNA was extracted using the RNeasy Mini kit (Qiagen, Hilden, Germany) according to the recommendations of the manufacturer. Yield and purity of the RNA extracts were photometrically measured and diluted to 20 ng RNA μl^{-1}. cDNA was prepared with 5 µl of each dilution, hexamers (1 µM) and Omniscript reverse transcriptase (Qiagen, Hilden, Germany) according to the protocol of the manufacturer. 1 µl cDNA of each transcript were added to 24 µl PCR mastermix (Quantitect SYBR Green PCR Mix, Qiagen, Hilden, Germany) containing either the primers Rp49 qF and Rp49-qB [17] or hymenopt F and B.[18] Real-time PCR was performed on a BioRad myiQ® device. Products of amplification were checked by analysis of melting curves and separation of the products either in agarose gels or on an automated gel device (QiAxcel, Qiagen, Hilden, Germany).

2.6 Residue analysis

Samples for chemical analysis of thiacloprid and its metabolite thiacloprid-amide were obtained from concentrated syrup stored in combs and from bee bread. Sampling was done mid September until mid October and at the end of March until mid May. Thiacloprid and its amide metabolite content in stored syrup and bee bread were determined by Bayer AG,[19] with modifications of the cited method. In brief, 0.5 g of the sample material was extracted in 10 ml methanol / water (3/1 v/v) and 0.1 ml L^{-1} formic acid. After centrifugation and concentration the aqueous solution was cleaned up on a Chromabond XTR™ column. Residues were eluted with 40 ml dichlormethane, dried, and collected in 2 ml toluene/ethyl acetate (85/15; v/v). After addition of 5 ml acetonitrile and 5ml toluene /ethyl acetate (85/15, v/v), the solution was passed through a 0.5 mg Silica Gel™ column. Residues were rinsed with toluene ethyl acetate (70/30, v/v), passed through the column again, eluted with acetonitrile/ water (95/5, v/v), dried and dissolved in 1ml methanol/water (1/9, v/v) and subjected to HPLC-MS/MS.

2.7 Parameters of colony performance

As parameters of colony strength, the number of brood cells and the number of adult bees per comb were visually estimated for each colony according to the "Liebefeld method".[20] Colony strength was assessed eight times per replication, four times before and four times after overwintering. The individual assessments per season (autumn/spring) were performed at intervals of 21 days ±1 day). On each assessment date the weight of the colonies was also determined using a digital balance. Box shaped wire grids with a mesh size of 8 mm were mounted in front of the entrances to collect dead bees and measure mortality. During the periods of bee flight, traps were emptied twice a week and the number of dead bees recorded. Prior to the first cleansing flight in

early spring, dead bees from the bottom boards of the hives were collected, weighed and counted to estimate the number of bees that died during winter. Winter colony losses and queen losses were recorded during regular inspections of the colonies.

2.8 Statistical evaluation

Statistical analyses were conducted with the software package SPSS version 20. Weights, numbers of bees and numbers of brood were checked for normality with the Shapiro –Wilks test, and for homogeneity of variances by Levene's test. Where required, data were subjected to transformation (square root or other as indicated in the respective figures) and analyzed with a repeated measurement ANOVA according to the glm repeated statement,[21] where group and year were considered as between subject factors. In the case of the brood values, no adequate transformation of the data was found. Nevertheless, repeated measurements ANOVA was conducted, as the group sizes were nearly equal, and therefore, violations of the assumptions normality of the data and homogeneity of variances were considered as not affecting the analysis.[22] The assumption of sphericity was tested with the Mauchly's test. In case of violation of the sphericity assumption the degrees of freedom were adjusted using the Box correction (in SPSS denominated as Greenhouse Geisser correction) [21] or by the Huynh and Feldt correction (if $\varepsilon \geq 0.75$). If a significant overall effect was found, groups were compared against each other with the Tukey test (if $\varepsilon \geq 0.75$; otherwise the Bonferroni procedure was used).[23] For comparing the parameter 'number of dead bees from the dead bee traps' the area under curves (AUC) were calculated and tested for normality. Where required, the values were log-transformed and subjected to ANOVA (proc univariate) for testing the differences between groups. The number of dead bees from the bottom

board of the hives in winter were square root transformed and subjected to ANOVA (proc univariate). For the parameter 'number of bees' a- power analysis was conducted with the software g*power.[24] To this end, the correlations between the measurements were Z transformed according to Rasch et al., (2014).[25] The required sample size for a medium effect with the effect size of f=0.25 was estimated with the g*power inherent procedure "ANOVA, repeated measures". As the data of *Nosema* spp had a skewed distribution with multiple zero values the non-parametric Kruskal Wallis test was used for comparisons. [26]

The Ct values of the real time PCR analyses for ABPV and the hymenoptaecin gene expression analyses were evaluated with the $2^{-\Delta\Delta CT}$ method relative to the controls of the first year.[27] Outliers within groups were identified and eliminated by Grubb's test (2 within of ABPV values and 4 within the rp49 values). Data were log (x+1) transformed to approximate variance homogeneity. Means of the groups expressed as fold- increase of ABPV loads relative to the control group of year 1 were compared with the help of glm univariate in SPSS. For the gene expression experiment no adequate data transformations to approximate normal distribution and homogeneity of variances were found. Therefore, outliers were identified by exploring the data as boxplots in SPSS. Datasets in which rp49 values were above the 75 percentile or below the 25 percentile were eliminated, as the outlying rp49 results were interpreted as failures in the extraction process of RNA. Kruskal-Wallis tests were performed to check for significant differences between the injected and the non-injected groups. In the case of multiple comparisons, alpha values were corrected according to Bonferroni.

3 RESULTS

3.1 Pathological characterization of the colonies

As the colonies were carefully treated for *Varroa* with coumaphos, the observed rates of mite infestation were low. The means averaged over all three years were 0.0316 mites g^{-1} bees for the C colonies (SD = 0.0453; N=90), 0.0332 mites g^{-1} bees for T1 colonies (SD = 0.0543; N=90) and 0.0395 mite g^{-1} bee for T2 colonies (SD = 0.0749; N=90). There were no significant effects of thiacloprid on the level of *Varroa* infestation (p_{THIA} =0.412; p_{year}<0.001). At the beginning of August the level of infestation ranged from 0 to 0.0581 mites g^{-1} bee. In mid-September the minimum was 0, the maximum 0.1691 mites g^{-1} bee and in October 0 to 0.4621 mites g^{-1} bee. After the application of coumaphos in winter 303.5 mites on average dropped from controls (SD= 254, N=30). From T1 colonies mean drops of 283.1 mites dropped on average (SD = 245.9; N=30) and from T2 colonies 261.8 mites dropped on average (SD = 187; N=30). The number of dropped mites did not differ significantly between groups (p=0.698, proc glm univariate), but was different for the factor year (p<0.001, proc glm univariate). The colonies were tested for viral infections. The PCRs of all samples were negative for chronic bee paralyis virus (CBPV). Deformed wing virus (DWV) was rare with 9 positives out of 84 samples detected 2 in controls, 2 in T1 colonies and 5 in T2 colonies. In contrast, acute bee paralysis virus (ABPV) was frequently detected. The factor treatment had no significant effect on the relative ABPV load (p=0.619). Differences between years were significant (p=0.003). The fold-values of ABPV were 2.5 for C (N=9), 16.1 for T1 (N=10) and 8.1 for T2 (N=10) in year 1, 95.0 (C; N=5); 55.9 (T1; N=8) and 2.1 (T2; N=9) for year 2 and 56.0 (C; N=9); 47.6 (T1; N=10) and 77.7 (T2; N=8) for year 3. Spores of *Nosema* spp. were detected, especially in the samples collected during summer. Control bees from end July / beginning of August contained

2.4 * 10^6. spores / bee (SD= 2.8 * 10^6, N= 30) vs. 2.0 * 10^6 spores / bee (T1 , SD= 2.1 * 10^6, N=30) and 2.1 * 10^6 spores / bee (T2 , SD= 3.2 * 10^6, N=30). Differences were not significant (p=0.761, Kruskal Wallis test). The loads of *Nosema spp.* in autumn and spring were lower. The respective mean values for autumn were 0.10 * 10^6; 0.10 * 10^6 and 0.11 * 10^6 (p=0.404 Kruskal Wallis test) and for spring 0.3 * 10^6; 0.0 * 10^6; 0.1 * 10^6 spores per bee (p=0.068 , Kruskal Wallis test).

3.2 Performance and strength of colonies

The parameters colony strength (number of adult bees), number of brood cells and weight gain were recorded for ten months per study replication. For the parameter adult bees the differences between groups were small and not statistically significant (fig. 1 – fig. 6; p_{THIA}= 0.113; p_{year}<0.001). With respect to brood significant differences were found (p_{THIA}= 0.037; p_{year}<0.001). Numbers of brood cells of C colonies and of T2-colonies were similar. Numbers of brood of T1-colonies were statistically significantly different from T2-colonies (p=0.032, Bonferroni procedure) but not from controls (p=0.811 Bonferroni procedure). Interactions between time of the measurements and treatment were not significant, for number of adult bees (p=0.125, Box correction), or for number of brood cells (p=0.061, Box correction). The values averaged over all eight estimation dates and all three years are shown in Table 1. In spring, colonies started the production of drone brood. Drone brood was recorded at four time points. Differences between groups were not significant (p=0.169; values were sqrt (x+1) transformed). However, the year was a significant factor (p<0.001). Interactions between the time of the measurements and the exposure to thiacloprid were not significant (p=0.127, Huynh-Feldt correction). The net weights of the colonies were similar, independent of

exposure to thiacloprid ($p_{treatmenat}$ =0.166; p_{year}<0.001). Mean net weight of 37.17 kg (N=237; SE= 0.612), 37.96 kg (N= 240; SE = 0.679) and 36.40 kg (N=238 SE = 0.609) were observed for the control, T1 and T2 treatments respectively. Interaction between time of the measurement and treatment were significant (p=0.009, Huynh-Feldt correction).

3.3 Estimation of the required sample sizes for detecting medium effects

For the parameter number of adult bees a high correlation between the repeated measurements with ρ = 0.86 (mean Pearson corr. coefficient) was found. Using the g*power inherent procedure "ANOVA: repeated measures" we calculated the total sample size which is required for detecting a medium effect of f=0.25 with a power of a 1-β error probability = 0.8 and an α error probability of = 0.05 (fig. 7). The total sample size amounted to 141 colonies. In the actual study design with three groups and three years 15.7 colonies are sufficient to verify a medium effect with an adequate power of 0.8.

3.4 Mortality of bees

Adult bee mortality in dead bee traps was regularly counted. In total, 1920 measurements were recorded in 2011/2012, 1726 measurements in 2012/2013, and 1937 measurements in 2013/2014. After aggregation by the AUC approach, 90 values were obtained for analysis of variance. The effect of the exposure of thiacloprid on the number of dead bees in dead bee traps was not significant (p=0.728, data log-transformed). The effect of the factor year was highly significant (p<0.0001). Interactions were not significant (p= 0.143). The respective means are shown in Table 2. At the end of each winter, shortly before the beginning of flight activity, the dead

bees on the bottom boards of the hives were collected and counted. On average, C colonies had 1027 dead bees per colony (SD = 744; N=30), T1 colonies 742 (SD = 623; N=30) and T2 colonies 719 dead bees (SD = 668; N=30). Differences between groups and years were significant (p_{year} <0.001, $p_{treatment}$ <0.001; data sqrt transformed, Interaction n. s. p=0.107).

3.5 Queen failure and colony survival

The exposure to thiacloprid had no effect on overwintering success. None of the colonies failed during winter. In each year, one queen out of the ten C- colonies and one queen out of the ten T2- colonies were lost. In 2011/2012 and 2012/2013 two queens of the ten T1-colonies failed. In the first year, all colonies survived until the end of the observation period. Spring failures occurred in 2013 (one T-2 colony) and in 2014 (one C- colony). Neither losses were replaced.

3.6 Residues of thiacloprid

Thiacloprid and its metabolite thiacloprid-amide were detected in syrup and in bee bread stored in the combs of the hives. In autumn, control colonies contained stored syrup with ~10µg kg^{-1} thiacloprid, treated colonies T1 ~150 µg kg^{-1}, and T2 ~780 µg kg^{-1} (Table 3). Before winter, concentrations of thiacloprid in the investigated matrices were slightly higher than after winter. In spring, residues were 12 µg kg^{-1} in C colonies, 94 µg kg^{-1} in T1-colonies and 631 µg kg^{-1} in T2-colonies, which demonstrates that the colonies were in fact exposed the whole time from the beginning of the experiment in July until spring. In autumn, the concentrations of thiacloprid amide ranged between 0.3 µg kg^{-1} (C) and ~8 µg kg^{-1} (T2) in stored syrup. In the treated colonies bee bread contained lower residue levels of thiacloprid and thiacloprid-amide compared to the

stored syrup. In spring, the concentrations in bee bread were similar to the values observed in autumn.

3.7 Expression of the hymenoptaecin gene

The constitutive expression and the challenged amount of mRNA of the hymenoptaecin gene were measured. Exposure to thiacloprid did not change the constitutive expression. However, after injection of *P. larvae* suspensions, bees exposed to thiacloprid expressed significantly lower levels of hym - mRNA than bees of the control groups (fig. 8).

4 DISCUSSION

Two groups of ten colonies each were exposed to different concentrations of thiacloprid by means of in-hive feeding of thiacloprid-spiked sugar syrup. Two concentrations, 0.2 mg L^{-1} thiacloprid and 2 mg L^{-1} thiacloprid were administered. Control colonies were fed with syrup containing the solvent acetone alone. The study simulated a long-term exposure with sub-lethal thiacloprid concentrations. The test was replicated three times in three subsequent years.

The dosages were chosen as to mimic a realistic field situation (T1 a lower level exposure group) or an unrealistically exaggerated exposure (T2 a higher level exposure group). Thiacloprid has been found in bee-collected pollen.[4] Pollen collected by foragers from treated apple flowers contained 0.03mg kg^{-1} of residues of thiacloprid.[28] Peak values of thiacloprid residues are reported from a Germany-wide survey (the German Bee Monitoring). A maximun concentration of 498 µg / kg was found in bee bread in 2012.[29] Concentrations in Austrian honeys varied widely between values below LOD and values up to 27.4 µg kg^{-1}.[5] Oilseed rape flowers treated with 50 g ha^{-1}

thiacloprid (PROTEUS 110 O™) had on average 6.5 µg kg^{-1} thiacloprid in nectar (Max. 208.8 µg kg^{-1} and median 2.5 µg kg^{-1}; 64 % positive samples) and 89.1 µg kg^{-1} thiacloprid in pollen (Max. 1002.2 µg kg^{-1} and median 4.1 µg kg^{-1}; 62 % positive samples).[30] Bee-collected nectar and honey contained around 2 µg kg^{-1} thiacloprid, whereas residues in bee-collected pollen and bee bread reached mean levels of up to 81.6 µg kg^{-1} thiacloprid.[30] A study from the US reports much lower concentrations in pollen (mean 23.8 µg kg^{-1}; ranging from 1.7 µg kg^{-1} to 115 µg kg^{-1} with 5.4 % positive samples out of 350, only [31]). Likewise, a recent Dutch study reports lower frequencies and lower residue levels in bee-collected matrices (bee-collected pollen < 66 µg kg^{-1}; honey: <15 mg kg^{-1} [32]). The heterogeneity of these findings can be explained by the different study designs, such as whether matrices of directly treated crops or of variable sources were analyzed, and by the different patterns of usage and thiacloprid-treated crops in different countries. There are no reports that describe findings of residue levels as high as 2 mg kg^{-1} thiacloprid in bee-relevant matrices under realistic exposure scenarios. The extremely long-lasting exposure over winter is not a field relevant scenario. Under practical conditions, beekeepers remove the honey in summer and feed the colonies with sugar syrup which is subsequently consumed during winter. Consequently, the high concentration of 2 mg L^{-1} and the extremely long duration of exposure of our study represent an unrealistic worst- case scenario.

The parameters colony strength, number of brood cells, production of drones, hive weight gain, overwintering success, and mortality were chosen as endpoints. According to EFSA,[33] colony strength is by far the most important and the most meaningful endpoint. In our experiment, no significant impact of the treatment on colony strength was observed. As the interaction between the factors time and exposure to thiacloprid

was not significant, there was also no delayed effect of thiacloprid on colony strength. Although the administered concentrations of thiacloprid were below the lethal level, a reduction in colony strength through sublethal effects might be expected, for such as reduction of the lifespan of the bees due to the long duration of exposure. Foragers leaving the hive supposedly consume thiacloprid containing syrup before departing. Several studies report effects of neonicotinoids on the orientation-, foraging and homing- capacity of honey bees,[8, 34-36] and several other sub-lethal effects of neonicotinoids are postulated which could lead to a depopulation of the colony even months after exposure.[37] The data on colony strength from our study do not support these hypotheses.

Perhaps even more important than the statistical significance of an experiment is the magnitude of the effects found.[38] According to EFSA [33], a reduction of the colony strength of 7 % can be considered negligible. Colonies of the T1 group were the strongest in absolute numbers of bees, averaged over all three years. A reduction of the mean strength of the control colonies by 7 % would correspond to a colony strength of 12230 bees. The mean number of the T2 colonies with 12277 bees was still within that range. Against this background, a potential effect of the thiacloprid treatment appears to be of insignificant magnitude.

Regarding the endpoint 'brood' differences between C colonies and the T1 colonies were not significant. However, the T2 colonies raised significantly less brood than the T1 colonies. The reduction of brood of T2 colonies was consistent over all three years. However, the brood cell numbers of the T2 colonies were not statistically significantly different from the control colonies. The respective values for T2 were 11969 brood cells

in year 1, 9699 in year 2 and 12312 in year 3 vs. 12851, 10259 and 14604 for the T1 colonies. Nevertheless, it has to be noted that the reduction of brood did not result in significant differences of colony strengths. To date there are no reports of a direct brood toxicity of thiacloprid, but for other neonicotinoids brood effects such as delayed development has been reported.[4] Indirect effects could also play a role, such as reduced olfactory capacities or constrained locomotory activity of nurse bees that could result in suboptimal brood temperature. The causality and the mechanisms behind the differences in brood production remain unclear. Here, too, interactions between time and exposure to thiacloprid were not significant, thereby the data do not support the hypothesis of a delayed impact on the production of brood. For thiamethoxam and clothianidin, Sandrock et al. (2014) [39] found a long-term reduction in the number of bees and of brood cells, possibly linked to the performance of the queens. In the case of thiacloprid, detrimental effects appearing long time after the exposure were not confirmed.

The endpoint weight development of the colonies was not affected by thiacloprid. All colonies were able to take up and to store the sugar syrup, draw out the wax foundation, and build up normal colonies of similar weights. The weight data did not provide any indication for impaired functionality of the colonies.

We did not observe increased mortality of colonies in winter, reduced survival of the queens, or impairment of the production of drone brood in spring. The reproductive capacity of the treated colonies in comparison to the control colonies was not affected. These observations do not support the conclusion of colony level effects of thiacloprid as derived from the study of van der Zee et al. (2015) [32]: this observational pilot study identified thiacloprid residues in honey and in bees as a significant risk factor for colony

collapse in winter. However, it remains unclear in this study whether the association between higher relative risk for overwinter survival and the presence of neonicotinoids was coincidental or causal. Other authors have reported strong impacts of the neonicotinoids thiamethoxam, clothianidin and imidacloprid on queen production and queen survival in *Apis mellifera*,[40] *Bombus sp.*[41-44] and on drone production in *A. mellifera*.[45] We did not observe similar effects of thiacloprid.

There was no increased acute mortality of adult bees in the thiacloprid exposed colonies. During the exposure period, the number of dead bees in the dead bee traps was similar in the treatment and the control colonies. However, the number of dead bees collected at the end of winter in the bottom boards did differ significantly between the three groups, with the highest numbers of dead winter bees observed in the controls. As thiacloprid has a moderate acute toxicity to bees, an elevated mortality concurring with the oral exposure could not be expected; interestingly however, even the long-lasting exposure during autumn and winter did not result in increased levels of mortality, either of individual bees or of colonies. As shown by the residue levels in the stored syrup before and after winter, the bees were in fact continuously exposed. Even though the content of thiacloprid in the body of the exposed bees was not measured in this study, the mortality data show that the bees were apparently able to prevent an accumulation of thiacloprid to toxic levels. The underlying mechanism is not yet fully understood. A metabolization of thiacloprid by way of the P450 detoxification system, by oxidization processes, or by excretion has been discussed.[3, 46, 47] In this context it is worth mentioning that the colonies were treated against Varroosis with coumaphos, and thereby had to cope with the double stress of simultaneous application of both synthetic drugs. Apparently, the body burden on the bees was such that they were able to tolerate

it which is in concordance with the literature. Synergisms between certain neonicotinoids and other pesticides are documented, but coumaphos and thiacloprid have been reported not to interact.[48, 49]

The concentrations of thiacloprid used in our experiment corresponded to a worst-case field-realistic scenario (T1) and a ten-fold increased unrealistic worst-case scenario (T2). Beyond that, we did not include additional extreme concentrations to enforce effects of an overdose of thiacloprid on honey bee colonies. While this could be seen as critical, there are, in fact, no operating procedures known to us which instruct to include positive controls in field assays with the goal to show the assay's sensitivity. Accordingly, no toxic standards are defined nor validated for field study designs. Toxic standards are not considered feasible in field trials, and positive controls are therefore not used, especially when the exposure can be shown by other means such as residue analysis, as done in our study, or observation of foraging activity in a treated crop. In fact, current testing guidelines do not recommend positive controls in field assays of pesticide testing.[50, 51]

EFSA requested that in pesticide testing field assays should generate data which can statistically be evaluated with a meaningful power.[52] Therefore we estimated the sample size which is required for detecting a statistically significant medium effect (fig. 7). Approximately 15 colonies per group and year were sufficient. Such rather high numbers of replicates raise the question of the practicability which should be considered in defining a regulatory study design.[53] Other guidelines state that the options for a statistical evaluation of field test data are limited. Limitation of statistical power is due to the relatively low number of replicates that are feasible and the possible violation of

the independence assumption.[51] The guidelines conclude that descriptive rather than interfering statistics might be appropriate. It was intended by the described study design of free ranging colonies exposed by in hive feeding to overcome these limitations as it allows handling a larger number of replicates. In our study ten colonies per treatment and year were each assessed eight times, resulting in a calculated total of 720 data sets (effectively 714 data sets as 6 data sets were not recordable due to colony failure). Despite the extensive number of data sets the power would not have satisfied the criteria according to EFSA (2013).[33] Further research on the improvement of the testing scheme may be helpful to overcome this issue.

In our experiment, the colonies were placed together in groups, and not distributed through the apiary following a random pattern. While it could be discussed whether this arrangement can truly be considered as independent replicates, this grouping was favored over a random pattern with the objectives to minimize the risk of carry-over of residues during colony handling and to minimize worker drifting between colonies from different experimental groups.[54] Drifting was probably not completely prevented as low amounts of thiacloprid residues were found even in the stores of control colonies. Similar pesticides shifts to the controls by drifting bees or from the outside were observed by others as well,[55] but nevertheless the studies are meaningful as the residues increased substantially in the expected directions.

In every field test, a balance has to be made between having all treatment units exposed to the same environmental factors, filtering out an important part of natural variability, and excluding potential influences between the different testing units. In our study we chose an approach which puts particular emphasis on the exclusion of the influence of

variable environmental factors by setting up all treatment groups together in one structurally homogeneous location. For instance, there were no trees that shaded a few colonies only, or any other evident environmental factors differentially influencing one of the groups. An alternative approach would have been to set up all colonies individually, in order to exclude interactions between the individual units. This would in turn have had the disadvantage that a higher number of replicate colonies would have been needed in order to compensate for the variability brought in by the differences between the different sites. In reality, however, due to increased time needed for the handling of the hives in many different locations, fewer replicates would have been possible due to logistic reasons. Overall, we consider the approach of exposure by in-hive feeding and placing ten colonies per group in a homogeneous test location a valuable design. It allows a controlled exposure and a statistical evaluation, even if small effects may not have been detectable as a result of the relatively low statistical power of the system. It has been suggested that power could be improved by increasing the number of replicates, which is, however, extremely difficult in practice due to reasons of experiment logistics, and /or by reducing the intrinsic variation e. g. with the help of better estimation methods.[56] Future studies should address the last mentioned aspect.

Pesticides can exert immuno-suppressive effects.[57] There are documented cases of immunotoxicity in insects.[58, 59] So far, little is known for honey bees. The exposure to multiple xenobiotics confronting honey bees in the environment has been discussed as a contributing factor to colony losses.[60] It is so far not well understood whether neonicotinoids can affect the functionality of the immune system of honey bees under practical field conditions. Clothianidin has been reported as an immunosuppressive

agent rendering bees more susceptible to viral diseases.[61] Other studies suggest that neonicotinoids increase the susceptibility to *Nosema* spp.,[55, 62, 63] which has also been reported for thiacloprid.[10, 11] Such effects have, however, so far not been observed under realistic field conditions in the context of entire bee colonies. The significance of the presumed interactions between pathogens and pesticides is therefore still under discussion. A recent study concludes that the interactions might be overemphasized.[64] According to Brandt et al (2016)[65] exposure of bees to thiacloprid can modify their numbers of hemocytes and their capacity to nodulate foreign objects. Hemocytes and nodulation are considered important constituents of the immune system of insects.[66] The study reported here compared bees from thiacloprid exposed colonies to control bees. As an immunity-related parameter, the expression of the gene encoding for hymenoptaecin (hym) was measured. This gene encodes the production of an antimicrobial peptide, which is effective in defending against bacteria.[67] It is easily quantifiable as the regulation of its expression can change up to two magnitudes.[68] The thiacloprid exposed bees showed a slight but statistically significant modification in the expression pattern. They produced less hymenoptaecin mRNA than control bees. The magnitude of effect was, however, low and we did not observe any impact on the colonies that may have been caused by this modification. The colonies were in a good health. The parasitization rates of *V. destructor* were low in all treatment groups and *Nosema* spp.infestations were within normal ranges. The loads of viral pathogens were without pathologically abnormal findings. As the colonies were initiated from treated shook swarms on virgin wax foundation with young queens, health problems were in fact unlikely. The pathogen burdens of control and treated colonies were of a similar low level. Therefore it is not possible to deduce from the findings of this study a

biological significance of immuno-modifying effects of thiacloprid at the colony level. Further research is required focusing on colonies suffering from pathogen and environmental stress, which is believed to aggravate the effect of pesticides.[55, 61, 62]

We demonstrated that healthy and well managed honey bee colonies are not adversely affected by a chronic exposure to thiacloprid at realistic and even at unrealistically exaggerated exposure levels. No adverse effects were observed, on either colony strength, overwintering capacity, or on the performance of the queens. Our study did not address the topic of potential synergisms between different pesticides or the performance of thiacloprid exposed colonies under poor health conditions. Future research could deepen our knowledge about these topics.

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Table 1. Number of bees, worker brood cells and drone brood cells. Shown are means, SE, and N of all three years of the experiment. Groups marked with different letters are significantly different (Tukey test).

Table 2. Number of dead bees collected in the traps fixed in front of the entrances.

Table 3. Residues of thiacloprid (THIA) and thiacloprid –amide in µg kg^{-1} found in stored syrup and bee bread of C colonies, of T1 colonies and T2 colonies. Given are means, min and max and Ns calculated over all three years.

Tab. 1

	Number of bees				Number of worker brood cells				Number of drone brood cells			
	Mean		**SE**	**N**	**Mean**		**SE**	**N**	**Mean**		**SE**	**N**
0 mg THIA L^{-1}	13151	a	448	237	11918	ab	636	237	3121	a	302	116
0.2 mg THIA L^{-1}	13645	a	421	240	12571	b	670	240	2846	a	281	120
2 mg THIA L^{-1}	12277	a	412	237	11344	a	623	237	2555	a	272	116

Tab. 2

	Mean year 1	**Mean year 2**	**Mean year 3**	**Overall mean**
0 mg THIA L^{-1}	26.55	21.28	11.69	19.84
0.2 mg THIA L^{-1}	25.90	25.02	10.22	20.38
2 mg THIA L^{-1}	25.13	22.15	12.01	19.76

Tab. 3

time		autumn		spring		autumn		spring	
group		Stored food				Beebread			
		THIA	THIA-amide	THIA	THIA-amide	THIA	THIA-amide	THIA	THIA-amide
C	mea	10.19	1.18	11.59	5.56	20.40	1.48	7.25	0.99
0 mg	n	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
THIA L^{-1}	min	48.00	2.26	65.62	13.10	304.21	3.70	44.40	1.31
	max	26	2	28	3	23	4	28	4
	N_{quant}	4	28	2	27	7	26	2	26
	$N_{<LOQ}$								
T1	mea	156.11	1.53	93.96	222	45.71	1.65	44.51	1.71
0.2 mg	n	33.81	<LOQ	1.36	<LOQ	9.64	<LOQ	8.50	<LOQ
THIA L^{-1}	min	1318.30	2.33	187.40	4.60	274.03	3.17	331.60	7.40
	max	30	29	30	29	30	4	30	22
	N_{quant}	0	1	0	1	0	26	0	8
	$N_{<LOQ}$								
T2	mea	777.76	7.77	630.57	13.42	203.28	615	205.89	4.36
2 mg	n	473.83	1.30	172.10	<LOQ	1.24	<LOQ	40.14	1.63
THIA L^{-1}	min	1278.20	12.44	882.40	21.10	369.1	14.65	441.00	12.78
	max	30	30	29	28	28	26	29	29
	N_{quant}	0	0	0	1	0	2	0	0
	$N_{<LOQ}$								

LOD = 0.0001 mg kg^{-1}

LOQ = 0.001 mg kg^{-1}

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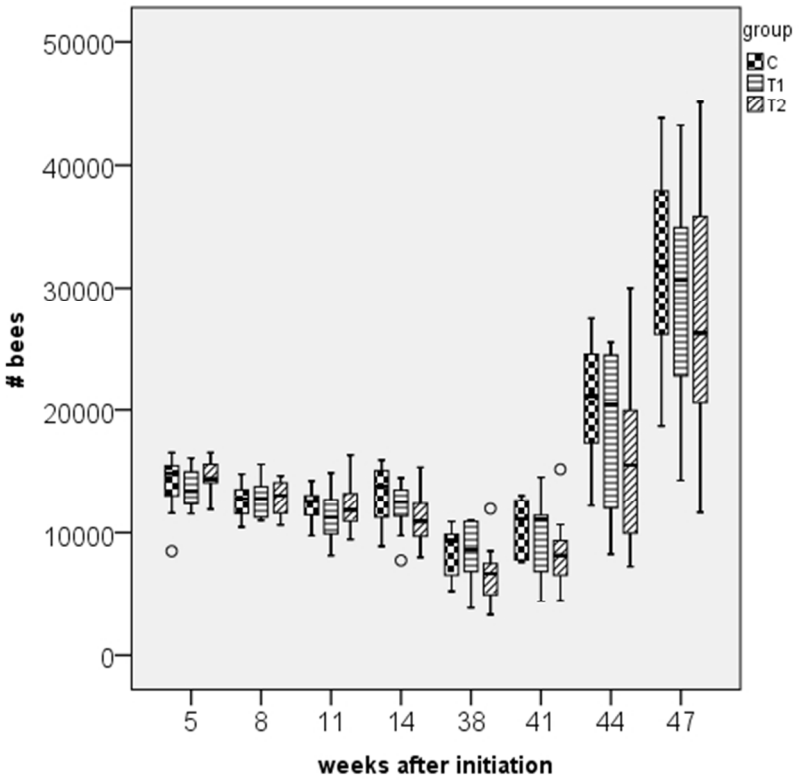

Figure 1 to figure 6: Number of adult bees and of brood cells for the three years and the three groups; N= 10 colonies per group; for bees: ptreatment = 0.113; p year< 0.001 and for brood ptreatment = 0.037, pyear<0.001 (glm repeated measurements). C: control colonies, not exposed to thiacloprid; T1: Colonies fed with syrup spiked with 0.2 mg thiacloprid L-1; T2: Colonies fed with syrup spiked with 2 mg thiacloprid L-1 ; Boxes labeled with '#': only 9 colonies of group T2 could be evaluated (1 not replaceable failure) and boxes labeled with '§': only 9 colonies of group C could be evaluated.
fig. 1: Bees in 2011 / 12
Overall differences between gr
220x176mm (72 x 72 DPI)

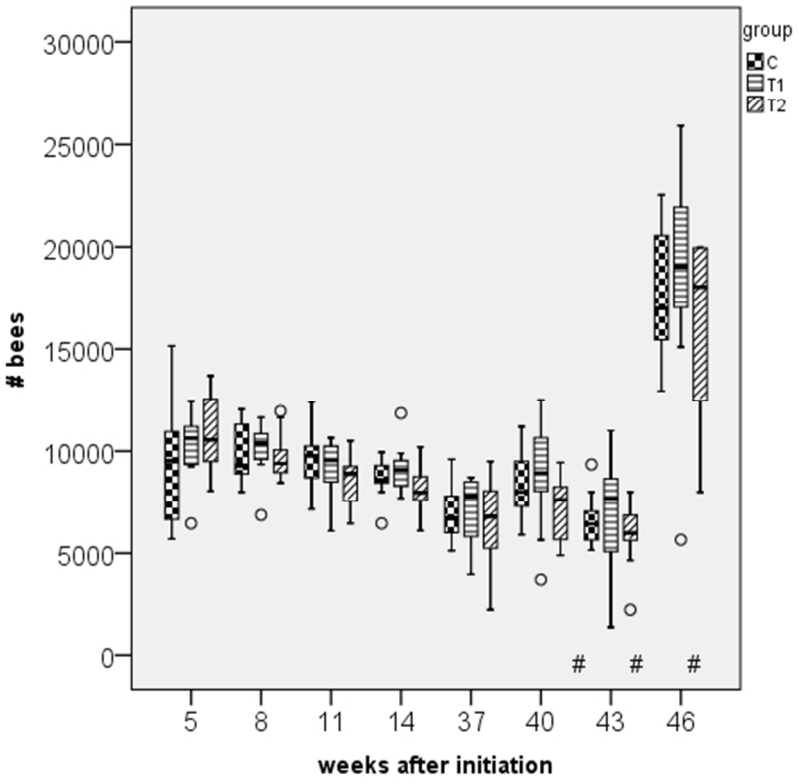

fig 2: Bees in 2012 / 13
Overall differences between gr
220x176mm (72 x 72 DPI)

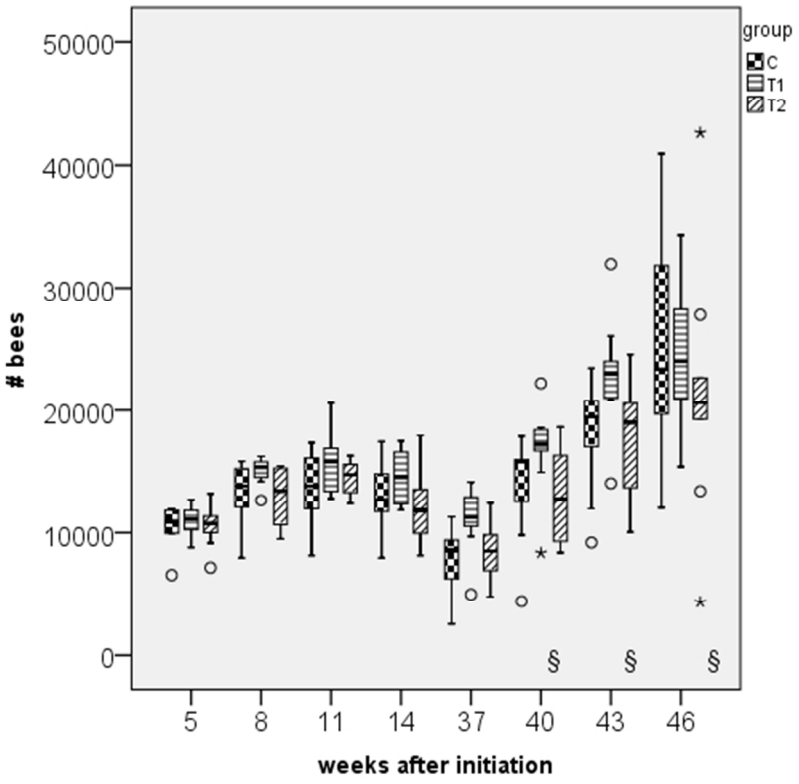

fig 3: Bees in 2013 / 14
Overall differences between gr
220x176mm (72 x 72 DPI)

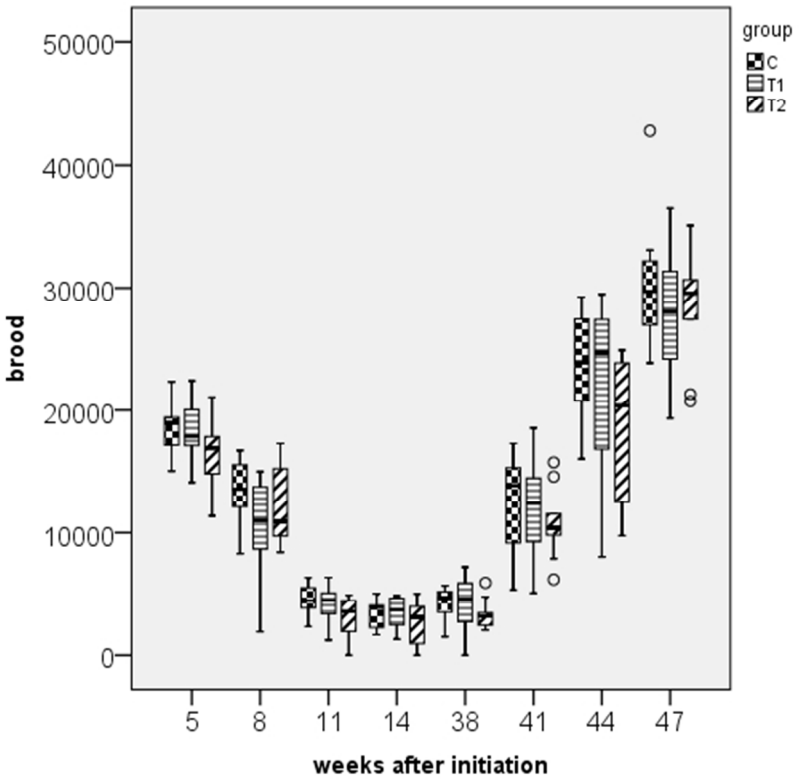

fig 4: Brood in 2011 / 12
Overall differences between gr
220x176mm (72 x 72 DPI)

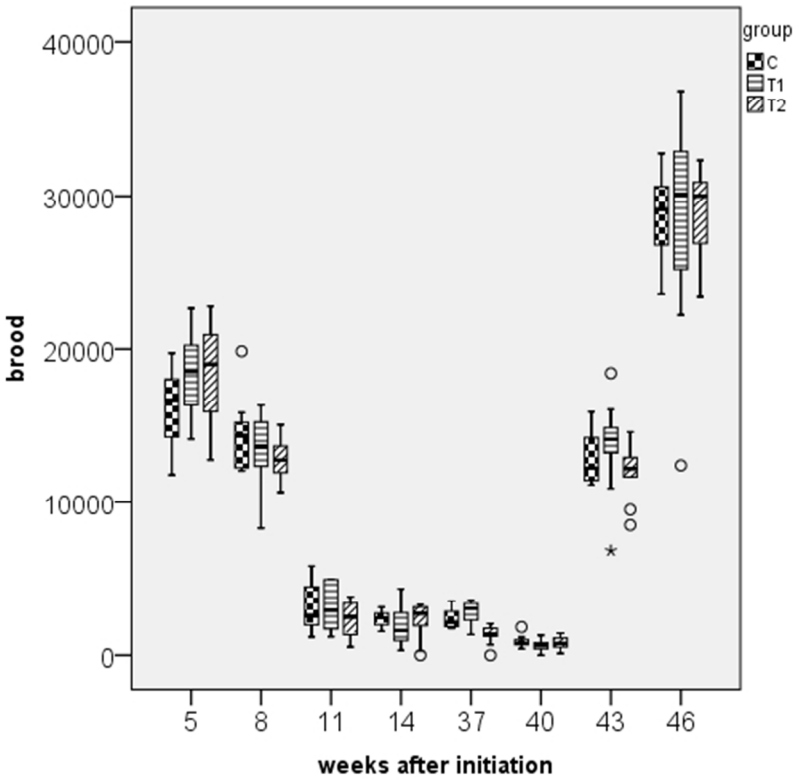

fig 5: Brood in 2012 / 13
Overall differences between gr
220x176mm (72 x 72 DPI)

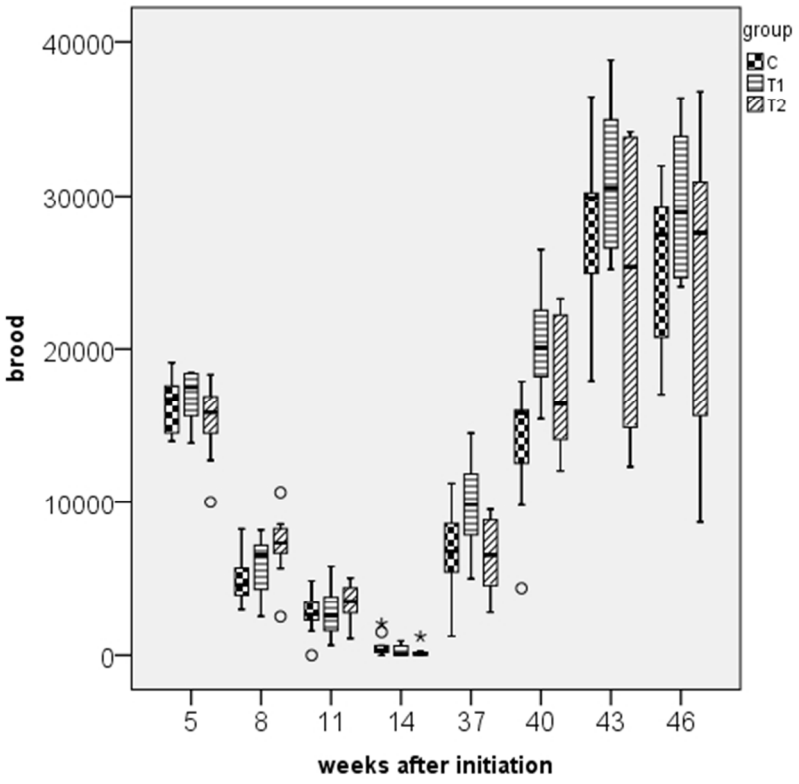

fig 6: Brood in 2013 / 14
Overall differences between gr
220x176mm (72 x 72 DPI)

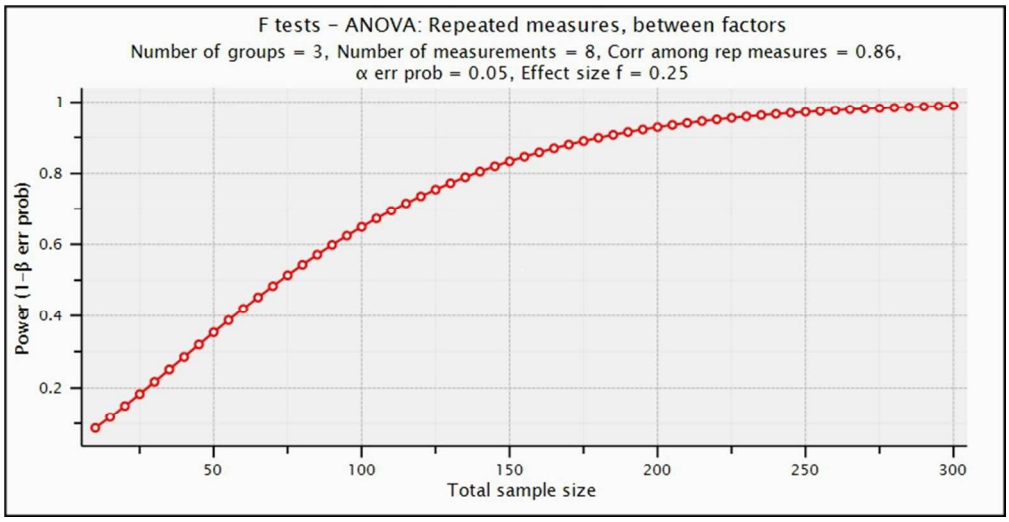

Figure 7. Total number of samples which are required to measure a medium effect of f = 0.25 for parameter 'number of bees' with α = 0.05 and β = 0.80. Power analysis performed with g*power.
we calculated the total sample
180x92mm (120 x 120 DPI)

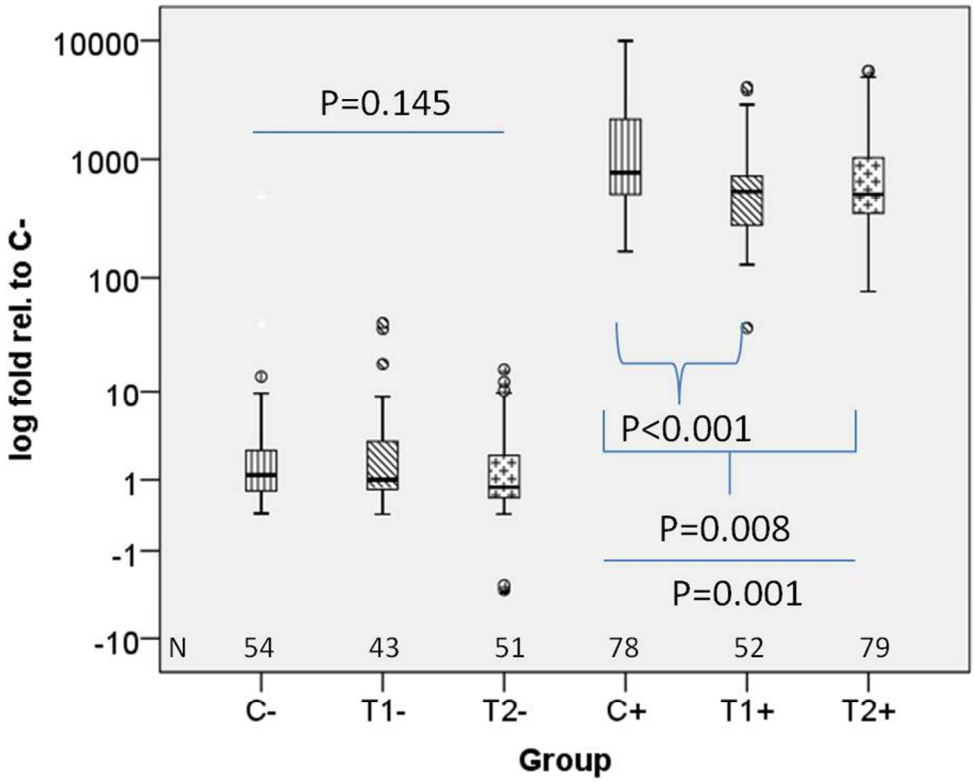

Figure 8. Expression of the hymenoptaecin gene relative to the controls of the first year without injection of P. larvae; on the left side: naïve bees without injection of P. larvae (marked with'-'), on the right side: bees which were challenged with P. larvae (marked with '+'), Kruskal Wallis test, pwith injection < 0.001; pwithout injection 0.145; multiple testing C+ vs. T1+ and C+ vs T2+,alpha = 0.025 Bonferroni corrected. N: number of tested bees.
However, after injection of P.
159x127mm (150 x 150 DPI)