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# A three-year large scale study on the risk of honey bee colony exposure to blooming sunflowers grown from seeds treated with thiamethoxam and clothianidin neonicotinoids

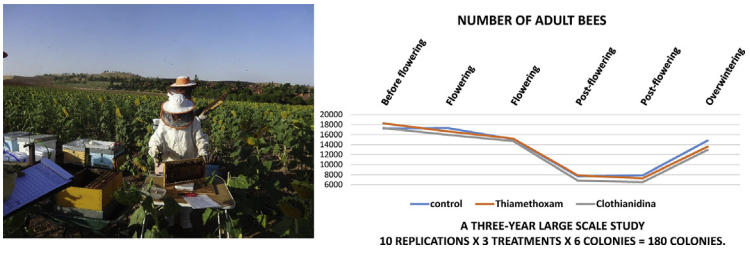
José M. Flores [a, *], Victoria Gámiz [a], Sergio Gil-Lebrero [a], Inmaculada Rodríguez [b], Francisco J. Navas [c], Ana I. García-Valcárcel [d], V. Cutillas [e], Amadeo R. Fernández-Alba [e], M. Dolores Hernando [d]

[a] Department of Zoology, University of Córdoba, Campus of Rabanales, 14071, Córdoba, Spain
[b] Department of Nutrition and Bromatology, University of Córdoba, Campus of Rabanales, 14071, Córdoba, Spain
[c] Department of Genetic, University of Córdoba, Campus of Rabanales, 14071, Córdoba, Spain
[d] National Institute for Agricultural and Food Research and Technology (INIA), 28040, Madrid, Spain
[e] Agrifood Campus of International Excellence (ceiA3), Department of Chemistry and Physics, University of Almeria, European Union Reference Laboratory for Pesticide Residues in Fruit & Vegetables, 04120, Almería, Spain

## HIGHLIGHTS

- This is a field realistic trials with 180 honey bee colonies and 10 replicates.
- Bees were exposed to sunflower blooms treated with thiamethoxam and clothianidin.
- No important effects, due to the neonicotinoids, were found.
- Honey bee colonies were the main factor for the result variability.
- Cohen'F test could explain the differences among laboratory and field researchs.

## GRAPHICAL ABSTRACT

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## ABSTRACT

Despite the restriction of the use of neonicotinoids in the EU, including thiamethoxam and clothianidin, the debate over their risk on honey bees has not been fully settled. This study presents results of a three-year study working with 180 honey bee colonies in ten replicates. Colonies were sorted into three treatments (60 colonies per treatment) exposed to sunflower blooms grown from seeds treated with thiamethoxam, clothianidin and a non-treated control. Each colony was assessed at six moments: one before to exposition to sunflower, two during the exposition (short-time risk), two after exposition (medium-time risk) and one after wintering (long-time risk). The health and development of the colonies were assessed by monitoring adult bee population, brood development, status of the queen, food reserves and survival. No significant difference among treatments when raw data was considered. However, when evolution from initial status of the colony was evaluated, a significant difference was observed from the first week of exposure to sunflower blooms. In this period, the number of adult bees and the amount of brood were slightly lower in the bee hives exposed to neonicotinoids, although such differences disappeared in subsequent evaluations. The concentration of residues in samples of beebread and adult bees was at the level of ng·g$^{-1}$. Magnitude of the effect of the treatment factor on the

* Corresponding author.
*E-mail address:* ba1flsej@uco.es (J.M. Flores).

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variability of colony health and development related parameters was low. The most important factor was the hive, followed by the replicate and year, and to a lesser extent the initial strength of the colonies.


## 1. Introduction

Some neonicotinoids, such as thiamethoxam and clothianidin have been identified as potential problems for honey bee viability (Lundin et al., 2015; Schmuck and Lewis, 2016), being banned for seed treatment in the EU (EFSA, 2018a and 2018b; European Commission Implementing Regulation No 784/2018, 2018a; European Commission implementing Regulation No 785/2018, 2018b). To date, the impact of neonicotinoids in field conditions has not yet been fully elucidated. Although several studies have been developed to test neonicotinoids, many of them reported contradictory results regarding toxic effects on pollinators. Contextualy, it is rather usual to find negative effects of neonicotinoids on bees in laboratory and semi-field trials, than in large-scale field trials (Johnson et al., 2010; Cresswell et al., 2012; Carreck and Ratnieks, 2014; Sanchez-Bayo, 2014; Sandrock et al., 2014; Goulson et al., 2015; Lundin et al., 2015; Blacquiere and Van der Steen, 2017; Carreck, 2017; Woodcock et al., 2017; Odemer et al., 2018).

In a preliminary paper (Hernando et al., 2018), we reported the results of the first year of a three years' study, performed on honey bee in field conditions. The effects of thiamethoxam and clothianidin applied in sunflowers seed were evaluated by means of a large-scale study. No effects on the honey bee colonies were observed as a result of the exposure to treated sunflower. In the present paper, we report the results of the three years of our research. Provided a significant effect of the initial state of the bee colonies on the results was detected in the preliminary paper, two statistical approaches were conducted, i) using raw data of variables regarding health status of the colonies and ii) the variation of those variables respect to initial status of the colonies.

## 2. Experimental

### 2.1. Study locations

The field study was carried out during three beekeeping seasons: 2015/16, 2016/17 and 2017/18. Each year, the study began in spring and ended in the early spring of the following year, covering a complete year cycle. Ten replications were scheduled: four in 2015, three in 2016 and three in 2017, in the Center and South of Spain (Fig. S1). In each replication, 3 plots were selected (one per treatment): sunflower crop (*Helianthus annuus*) grown from untreated seeds (CO), seeds treated with thiamethoxam (Syngenta's Product Cruiser® FS 350 g/L, at a rate of 0.25 mg a.s./seed) (TH), and seeds treated with clothianidin (Bayer's Product Poncho® FS 600 g/L, at a rate of 0.5 mg a.s./seed) (CL). Seeds of Bosfora® variety were used during the first year. To lengthen the bloom period, Vento® and Damascus® varieties (Syngenta) were also sown during the second and third year. The three varieties were sown in alternating parallel lines. To tackle the use of commercial fields for experimental studies of PPP under restriction by EU regulation, the approval by the Competent Administration Authority was required. The design of field study was described in Hernando et al. (2018), where the risk framework for evaluating neonicotinoids was considered, whenever possible (EPPO 170a, EFSA, 2013). This includes 2-ha plots, separated at least 2 Km when possible, isolated from other crops or blooms attractive to bees (Fig. S1). The plots were randomly assigned to each treatment, except for the cases when crops could not be place more than 2 km apart. In such cases, the control plot was the most distant from the treated plots. In each replication, the plots of the three treatments were sown the same day, and flourished simultaneously. Hives were placed in crops when at least 20% of plants were at the phenological growth stage 61–62 of BBCH scale ("Beginning of the flowering: ray florets extended, disc florets visible in outer third of inflorescence") and they were removed in stage 69 ("End of flowering: most of the disc florets have finished flowering, ray florets dry or fallen") (Biologische Bundesanstalt, Bundessortenamt and Chemical Industry) (Meier, 2001).

### 2.2. Honey bee colony handling and management

For each treatment and replication, 6 colonies were evaluated (180 colonies). Handling and management is described in Hernando et al. (2018). In brief, the bee hives were kept in a Mediterranean forest area, except during sunflower bloom. Swarms of *Apis mellifera iberiensis* (adult bees, brood and reserves), were obtained from professional beekeepers and housed in Langstroth hives, with screening bottom boards. All swarms were requeened with marked queens reared in spring, with free mating. During five week, the swarms were fed with sugar syrup and nutritional supplement for bees. For the growth of swarms, comb foundations were supplied. Natural pollen harvesting was sufficient. The colonies were provided with water sources in nearby areas at all the time. The bee hives were managed as in standard professional beekeeping.

### 2.3. Evaluation of the colony status and health

The colonies were evaluated six times (CE1 to CE6), coinciding with critical moments of development and exposure. CE1 was just before hives were moved to sunflower fields, being the starting point to determine the initial status of colonies. Based on the colony status, determined in CE1, bee hives were homogenously distributed between 3 groups of 6 hives in each replication. Each group was then randomly assigned to each treatment. To ensure exposure of bees to sunflower and that the bees mostly ate the food collected from the blooms, we wanted prevent mixing of pollen and honey of sunflower with what was previously stored. Thus, combs with existing reserves were replaced by comb foundations from organic beekeeping in the first year. However, since a colony can fail due to lack of food (Hernando et al., 2018), only two combs of each bee hive were replaced in the second and third years.

Additionaly, the adult bee population recorded in this evaluation was used to establish the initial strength of the colonies. With this criterion, bee hives were grouped in quartiles as part of the statistical analysis.

CE2 was a week after introducing hives into the sunflower fields; CE3 two weeks after, just before being removed and taken back to the apiary located in the forest of Córdoba. Given the exposure time, CE2 and CE3 could record potential short-term effects. CE4 took place at the end of summer, and CE5 in early winter. CE4 and CE5 served to record medium-term effects, after hives were removed from blooms. CE6 was in spring of the following year

to evaluate potential long-term effects. In each evaluation, the following parameters were recorded: colony strength (estimated by the number of adult bees per bee hive), brood development, queen status, diseases and food reserves. The methodology for evaluating such parameters related to colony health and development was described in Hernando et al. (2018). In brief, the population of adult bees (PAB) was determined by weighing the bees in the swarm, brushed from the combs, and obtaining the average weight of each bee, from a sample of approximately 10 g.

Photographs of each frame were analyzed with an image analysis program (Image J®) for determining brood, pollen and honey areas. Also, queen status and disease symptoms were recorded. In addition, *Varroa destructor* is considered the most devastating parasite of colonies. In this regard, the application of veterinary treatments is mandatory in the Spanish regulation (Royal Decree 608/2006). For the control of Varroa, Amicel® (amitraz, 0.5 g) was administered.

In CE3, when the highest concentration of residues should be found in bee hives, and CE6 at the end of the test, for residues analysis, samples of adult bees (approx. 10 g, collected from the bottom of the beehive, with a higher proportion of foragers) and 3 pieces of combs (approx. 25 $cm^2$) with brood, bee bread and honey respectively, from each colony were collected. Additionally, samples of honey were taken in CE2 and CE3 for the melisopalinological analysis of the honey collected during bloom exposition. We only considered that an effective sunflower exposure had occurred when the bees had stored monofloral honey with more than 23% sunflower pollen. This decision was taken given literature suggests a minimum of 20% may be required (Terrab, 2003) and provided we wanted to maintain the 23% minimum recorded in the first year of evaluation (Hernando et al., 2018).

Detailed information on the methodology is shown in supplementary material "Evaluation of the colony status and health" and "Analysis sampling".

### 2.4. Analysis of neonicotinoid residues

Quantification of residues was based on LC-QqQ-MS (liquid chromatography – triple quadrupole - mass spectrometry) analysis. An UPLC 1290 Series coupled to a 6490 LC/MS (Agilent Technologies, Palo Alto, CA, USA) was used. The system has a JetStream electrospray ion source. Additional analysis were based on LC-Q-Orbitrap-MS. An UHPLC Dionex™ Ultimate 3000 (Thermo Scientific™, San Jose, USA). A QExactive Focus (Thermo Scientific, Bremen, Germany) mass spectrometer was equipped with Heated Electrospray Ionization Source (HESI II). Samples of bee bread and adult bees collected in CE3 were analyzed. The extraction procedure and analyses by LC-QqQ-MS and LC-Q-Orbitrap-MS are described in supporting information (Hernando et al., 2018; Hakme et al., 2017; Vázquez et al., 2015).

Method validation was performed following quality control standards (ISO 17025). Identification of residues was based on acquisition of 2 SRM (selected reaction monitoring) transitions, retention time (tolerance ±0.2 min) and SRM ratio compliance ($SRM_2/SRM_1$, tolerance ±30%). For quantitative analysis, we used standard addition involving measurement of the analyte signal in a sample extract versus spiked extract, and for determining ion suppression or enhancement, as result of matrix complexity.

### 2.5. Data analysis

The statistical model was a multivariate animal model with simple observations including hive weight, bee population, brood, reserves of pollen and honey. Fixed effects comprising the mixed model consisted of year, Y (1, 2 and 3); treatment, T (CO, TH and CL); replicate, R (1–10); quartile, Q (1, 2, 3 and 4) and colony evaluation, CE (CE1-CE6) and the random factor of bee hive, B (1–173) as suggested by Bate and Clark, 2014. In function notation, mixed multivariate model was:

$$Y_{ijklmn} = \mu + Y_i + T_j + R_k + Q_l + CE_m + B_n + \epsilon ijklmn$$

where $Y_{ijklmn}$ is the separate score of variables related to the development and colony viability for a given bee hive; $\mu$ is overall mean; $Y_i$ is the fixed effect of ith year of evaluation (i = 1, 2, 3), $T_j$ is the fixed effect of jth treatment (j = CO, TH, CL); $R_k$ is the fixed effect of kth Replicate (k = 1 to 10); $Q_l$ is the fixed effect of lth quartile (l = 1 to 4); $CE_m$ is the fixed effect of mth evaluation (m = CE1, CE2, CE3, CE4, CE5, CE6); $B_n$ is the random effect of nth bee hive, and $\epsilon$ijklmn is the random residual effect.

To determine which factors may be considered fixed or random, we followed the descriptions in Crawley (2012). According to this author, the difference between fixed and random effects is that fixed effects have informative factor levels, while random effects often have uninformative factor levels. This means random effects have factor levels drawn from a large population in which the individuals or units differ in many ways, but we do not know exactly how or why they differ (that is random effects comprise an undetermined number of levels). As initial colony strength has a significant effect on the assessed variables (Hernando et al., 2018), two approaches were conducted using raw data of variables regarding health status and the variation of those variables respect to initial status. In a first approach, data directly obtained from bee hives was assessed to confirm and expand the results from the first year (Hernando et al., 2018) to three years. Second, percentage of variation occurring on each evaluation respecting to the initial status (CE1), previous to the introduction of hives in crops was computed using original data.

$$\%\ variation\ between\ nth\ CE\ and\ 1st\ CE = \frac{(nth\ \text{CE variable value} - 1\text{st CE variable value})}{1\text{st CE variable value}} * 100$$

nth is the n position of CE in the sequence of CEs (1–6) in the study.

Original data from three years was normally distributed, for all variables (Kolmogorov Smirnov, $P > 0.05$) except for pollen at CE4 and CE5, and honey for all evaluations. ANOVA and Tukey's HSD post-hoc test were performed to determine whether there were any significant differences across the three treatments for the variables considered in the model. For the study of the percentage of variation with respect to first evaluation, as parametric assumptions were not met, Kruskal–Wallis H test was performed to study potentially existing differences between-levels of T within the same independent variable. The Kruskal-Wallis test calculates probability values based on a chi-square approximation (Schmoldt, 1993).

Afterwards, we studied the pairwise comparisons for treatment pairs for which a significant effect had been reported using the Dunn's test (which systematically performs Mann Whitney's test for all possible pairwise comparisons). If we test for multiple comparisons (hypotheses), the likelihood of incorrectly rejecting a null hypothesis increases, that is rejecting the existence of statistically significant differences between two or more groups (Type I error). The Bonferroni correction was applied as it compensates for that increase. All nonparametric tests were carried out using the independent samples package from the non-parametrical task of SPSS Statistics for Windows, Version 24.0, IBM Corp (2016).

Cohen's f was computed for all possible combinations of the factors and quantitative variables presented above as it allows to analyze the relationship between continuous and categorical

variables in the case where the latter has more than two possible levels (k values). As suggested in Field (2000), Cohen's F is a good choice to perform after ANOVA or Kruskall Wallis (with Bonferroni corrrection) to describe the average effect across as many levels (k levels of a categorical or nominal variable) as considered per factor provided the high interpretability of this parameter. The value of the Cohen's f will always be $\geq 0$. The greater the value of Cohen's f, the more intense association between variables will be. Cohen's f is a good example of a standardized effect size measurement as it divides size of the effect by the relevant SD.

SPSS cannot calculate the Cohen's f directly, but they can be calculated using partial eta$^2$ ($\eta p^2$). Cohen analyzes the relationship between f values of Cohen and partial Eta$^2$:

$$\eta^2 = \frac{f^2}{\left(1+f^2\right)},$$

$$f = \sqrt{\frac{\eta^2}{(1-\eta^2)}}$$

where $f^2$ is the square of effect size, and $\eta^2$ is to $\eta p^2$ calculated by SPSS (Cohen, 1988). While Cohen's f can be benchmarked against Cohen (1988) criteria of small (0.1), medium (0.25), and large (0.4) effects, $\eta p^2$ can be benchmarked against Cohen (1988) criteria of small (0.01), medium (0.09), and large (0.25) effects as suggested in Richardson (2011).

## 3. Results and discussion

It should be noted that seven of the 180 colonies were excluded from data processing, because the melisopalinological analysis showed that the criteria of 23% in pollen was not reached in CE2 and CE3 (Table S1). The average percentage of sunflower pollen was $38.75 \pm 10.81$, $38.09 \pm 12.42$, and $36.58 \pm 9.99$ for the colonies of CO, TH and CL, respectively. No significant differences were detected (Kruskal-Wallis Test, $p > 0.05$).

### 3.1. Statistical analysis and effect evaluation of the effect of the factors

When considering raw data of variables regarding colony status for three years, findings proved to be very similar to those found for the first year (Hernando et al., 2018). There was no significant difference across treatments in the evolution of the PAB or in hive weight for any of the evaluations (one-way ANOVA, $p > 0.05$). PAB and brood decreased during the remaining blooms until the end of the summer and increased after autumn. During CE2, brood area was significantly higher in CO than in CL (one-way ANOVA, $p < 0.05$. Tukey's HSD Test, $p < 0.05$). In CE3 and CE4, TH presented significantly higher pollen reserves than CL (Kruskal-Wallis test, $p < 0.05$. Mann-Whitney test, $p < 0.05$). Bee hives exposed to TH presented largely increased honey reserves at the end of blooms, although such difference was not significant among treatments (Kruskal-Wallis, $p > 0.05$). A summary of the descriptive statistics for the raw data considered in the study is presented in Fig. 1 and Table S2.

When the percentage of variation of each particular evaluation (CE2 to CE6) with respect to the first evaluation (CE1), a significantly higher weight was shown in TH than in CL in CE3. However, no significant difference was observed across any of the evaluations when CO was considered (Kruskal-Wallis Test, $p > 0.05$. Mann-Whitney test, $p > 0.05$) (Table S3). Additionaly, a significant reduction in PAB in CE2 was also detected. The magnitude of such reduction was higher in TH and CL than in CO (Kruskal-Wallis test, $p < 0.05$. Mann-Whitney test, $p < 0.05$) (Table 1 subparagraph B Table S4). Contrastingly, no significant differences were detected across the rest of evaluations (Kruskal-Wallis Test, $p > 0.05$).

A higher reduction in brood area was also observed in CE2 in TH and CL when compared to CO. Such effect disappeared later, since no significant difference was detected across treatments in evaluations CE3 to CE6 (Kruskal-Wallis Test, $p > 0.05$. Mann-Whitney test, $p > 0.05$) (Table S5). During CE4, hives exposed to TH had significantly greater pollen reserves than those exposed to CL and in CO (Table S6) (Kruskal-Wallis Test, $p < 0.05$. Mann-Whitney test, $p < 0.05$). This was also observed for honey at the end of blooms, although such difference was not significant across treatments (Test of Kruskal-Wallis, $p > 0.05$) (Table S7).

When all the dataset was considered, from the moment in which the colonies were exposed to the blooms until the end of the study (CE2 to CE6), Chi-squared ($\chi^2$) revealed significant differences for PAB, brood, reserves of pollen and honey across the years of evaluation. While significant differences for all the variables across replicates were detected, for treatment, a significant difference for PAB, hive weight, and pollen. Table 2 and Supplementary Tables S8–S10 report a summary of the results for Kruskal-Wallis H tests for the colony, quartile, hive and location, Cohen's f confirms the empirical appreciation throughout CE1-CE6, with a high influence of hive factor (Table 2). It refers to the colony as a whole, considering queen status, the composition of subfamilies of sister bees that constitute colony, with own genetic and differentiated response to stimuli, quantity and quality of food reserves and health status (e.g., Varroa population). This makes the colony a superorganism for which it is really difficult to achieve the homogeneity reached in controlled laboratory trials. Contrastingly, this diversity enables a great flexibility of the colonies to successfully face adverse conditions, which would be known as resilience of the colony (Thompson et al., 2019). Regarding to location, Replicate mainly comprises the bloom status and may affect both the accumulation of reserves and the cost in bees resulting from an increased foraging activity (Khoury et al., 2013). Location influenced bee hives even after these were removed from sunflower until CE6. Climatic conditions during exposure were similar, not only between plots, but also between replicates. Year of evaluation influenced on the variability, which could be explained by distinct climatic conditions, blooms and use of different bee hives. Influence of Treatment on the variability in the variables, was low or nonexistent, being somewhat slightly higher during exposure, and decreasing or disappearing later (Table 2). In any case, the effects of Treatment could be either positive or negative. For example, Treatment effect was positive for pollen reserve, which was the only variable to be affected by Treatment in all the evaluations. Bee hives exposed to TH had higher pollen reserve in CE4. On the other hand, since increased harvesting efforts can negatively affect the adult bee population (Khoury et al., 2011), we speculate that differences in PAB could be attributed to the crop conditions and their exploitation by bees, rather than to eventual effects of Treatment.

In Cohen's terminology, a value $< 0.1$ indicates that the effect can be considered negligible, from 0.1 to 0.24 indicates a small effect size, which means an effect to be assessed after careful study; $\geq 0.24$ is an effect big enough, and/or consistent enough, that could be appreciated 'with naked eye' (Cohen, 1988). This could explain the contradictions found between results obtained in laboratory and field trials. It is possible that, if there is an effect of thiamethoxam and clothianidin on bees, it is so small that can only be detected after using unrealistic exposure rates, together with intricate experimental design, as findings Wood et al. (2020). Such experimental conditions are difficult to implement in field conditions. On the other hand, the focus organism should be the whole of the colony and its resilience, and not bees individually (Thompson et al., 2019).

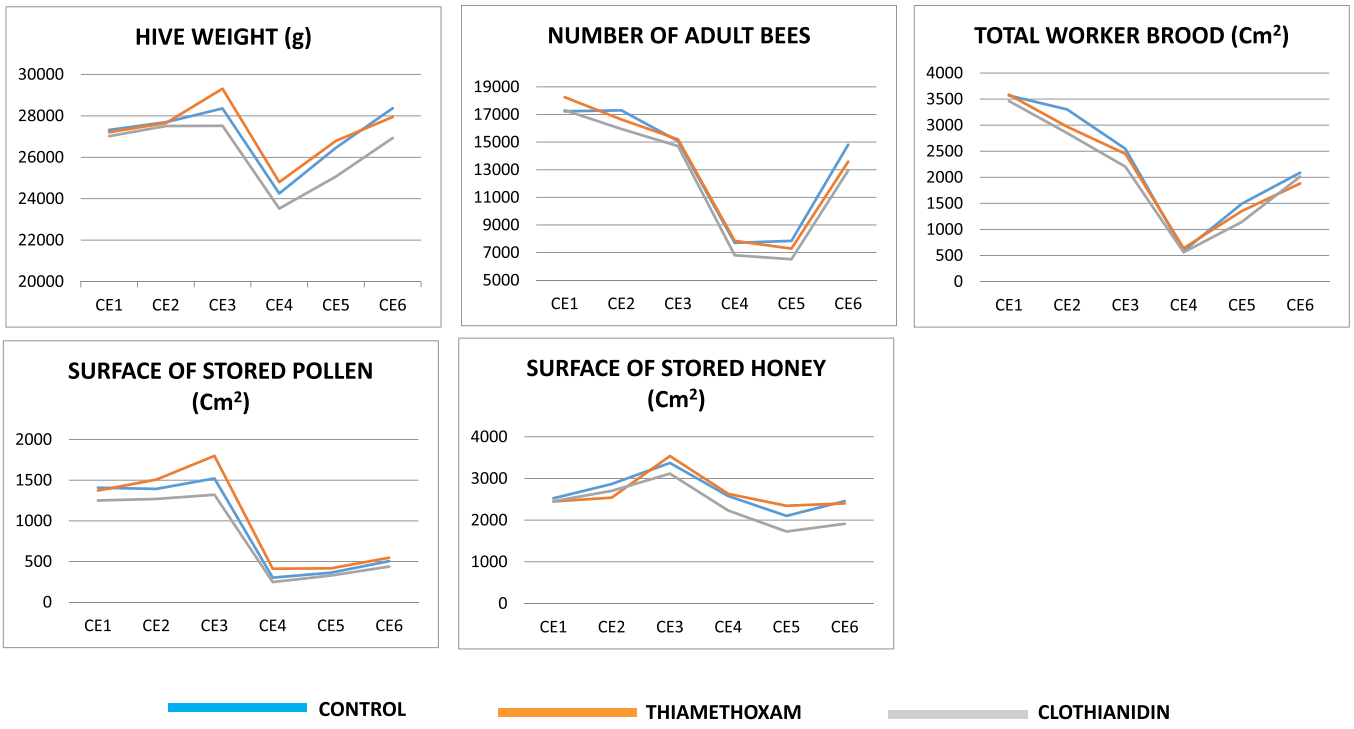


**Fig. 1.** Evaluation of the health and development of the colonies by monitoring hive weight, adult bee population, brood area and reserves of pollen and honey areas. The graphics are constructed from the original raw data recorded in the bee hives. Data is presented as average values of the colonies of each treatment in each of the colony evaluations (CE1-CE6). More detailed information in the supplementary material (Table S2).

### 3.2. Colony health and development

The change in PAB is a determining variable in the evaluation of toxic effects (EFSA, 2013). A colony is a superorganism where bees, as social individuals, establish complex relationships for the development of their functions, so a change in PAB can only be reliably measured in field or semi-field trials. Unfortunately, these are the least frequent trials, due to the costs and complexity involved (Lundin et al., 2015). Different studies have been carried out to evaluate the effects of the thiamethoxam and the clothianidin on adult bees, with conflicting results. For example, in maize and oil seed rape. Sandrock et al. (2014), Siede et al. (2018) and Thompson et al. (2019) found negative effect on honey bees due to these neonicotinoids, directly administered to artificially fed bee hives in semi-field conditions. By contrast, Cutler et al. (2014) and Rolke et al. (2016), under landscape-scale conditions, did not detect significant negative effects on bees exposed to treated crops. Pilling et al. (2013) found a low risk for bee colonies. While Woodcock et al. (2017) found contradictory results, positively or negatively affecting bee populations according to the country where trials were performed. Our findings in sunflower crop, show that in CE2, there was a decrease in PAB due to TH and CL, while in CO, PAB maintained. Subsequently, in CE3, CO group decrease in PAB, and differences among treatments disappear (Fig. 1; Table 1 subparagraph A; Tables S2 and S4). A cause could be a toxic effect of the treatments, but this should lead to the increase of differences in

**Table 1**
Percentage of variation (%) in the population of adult bees in the colonies throughout the six colony evaluations (CE2 to CE6), referencing the initial value in CE 1 as 100%. The data are presented as a percentage of variation (mean ± SD) of each evaluation with respect to the initial evaluation **(A)**, variation points of the percentage of each evaluation with respect to the previous evaluation **(B)** and points of variation of the colonies of the treatments with thiamethoxam and clothianidin with respect to the control group **(C)**.

| **A. Percentage of variation in the population of adult bee of each treatment, for each evaluation with respect to the initial state of each colony (CE1).** | | | | | | |
|---|---|---|---|---|---|---|
| **Treatments** | **CE1** | **CE2** | **CE3** | **CE4** | **CE5** | **CE6** |
| **Control** | 100 | 101.7 ± 16.4 | 89.1 ± 19.6 | 45.7 ± 15.7 | 45.2 ± 20.8 | 87.1 ± 42.9 |
| **Thiamethoxam** | 100 | 93.6 ± 17.9 | 84.9 ± 18.2 | 45.2 ± 17.4 | 43.8 ± 28.9 | 83.2 ± 60.9 |
| **Clothianidin** | 100 | 92.9 ± 13.6 | 85.4 ± 22.8 | 39.6 ± 13.7 | 37.3 ± 22.4 | 75.6 ± 47.6 |
| **B[a]. Points of difference in *nth* in the population of adult bees of each treatment and evaluation with respect to the initial state (CE1).[a]** | | | | | | |
| **Treatments** | | **CE1 to CE2** | **CE2 to CE3** | **CE3 to CE4** | **CE4 to CE5** | **CE5 to CE6** |
| **Control** | | 1.7 | −12.6 | −43.4 | −0.5 | 41.9 |
| **Thiamethoxam** | | −6.4 | −8.7 | −39.7 | −1.4 | 39.4 |
| **Clothianidin** | | −7.1 | −7.5 | −45.8 | −2.3 | 38.3 |
| **C. Points of difference in *nth* in the population of adult bees of the treatments with thiamethoxam and clothianidin with respect to the controls in each of the evaluations.[a]** | | | | | | |
| **Thiamethoxam** | | 8.1 | 3.9 | 3.7 | 0.9 | 2.5 |
| **Clothianidin** | | 8.8 | 5.1 | 2.4 | 1.8 | 3.6 |

[a] Positive values indicate increments; negative values indicate negative differences.

**Table 2**
Cohen's f effect power of the factors considered on the variables studied in bee hives. Cohen's f test for the size of the effect of categorical variables on continuous variables. This test measures the effect of the factors on the variability recorded in the variables. Values up to 0.1 mean the effect is considered negligible. Values between 0.1 and 0.25 are considered indicative of a low effect. Values between 0.25 and 0.4 are indicative of a moderate effect. Finally, greater than 0.4 indicates a strong effect. For values that are not shaded, chi-squared ($\chi^2$) did not reveal significant differences between levels. In this case, values are calculated from the *nth* of the variables, compared to the levels of those variables in CE1 (prior to introducing the hives in the sunflowers). The data are presented for the whole data set (A), considering only the period of exposure to the sunflower blooms (B), or considering the period after the removal of the hives from the sunflower blooms (C). For more complete information, see Supplementary Information Table S8, Table S9 and Table S10.

| A | Annuity | Location | Treatment | Hive | Quartile |
|---|---|---|---|---|---|
| Weight % dependent | 0.110 | 0.632 | 0.098 | 0.815 | 0.120 |
| Population of adult bees % dependent | 0.200 | 0.234 | 0.086 | 0.504 | 0.233 |
| Population of brood % dependent | 0.179 | 0.213 | 0.083 | 0.958 | 0.116 |
| Reserves of pollen % dependent | 0.160 | 0.223 | 0.124 | 0.773 | 0.126 |
| Reserves of honey % dependent | 0.363 | 0.882 | 0.045 | 10.091 | 0.097 |
| **B** | **Annuity** | **Location** | **Treatment** | **Hive** | **Quartile** |
| Weight % dependent | 0.358 | 1.379 | 0.096 | 2.348 | 0.206 |
| Population of adult bees % dependent | 0.118 | 0.239 | 0.156 | 1.595 | 0.307 |
| Population of brood % dependent | 0.165 | 0.234 | 0.104 | 2.351 | 0.112 |
| Reserves of pollen % dependent | 0.245 | 0.348 | 0.153 | 1.916 | 0.160 |
| Reserves of honey % dependent | 0.564 | 1.584 | 0.005 | 3.123 | 0.099 |
| **C** | **Annuity** | **Location** | **Treatment** | **Hive** | **Quartile** |
| Weight % dependent | 0.346 | 0.699 | 0.117 | 10.305 | 0.145 |
| Population of adult bees % dependent | 0.418 | 0.470 | 0.100 | 10.109 | 0.265 |
| Population of brood % dependent | 0.242 | 0.281 | 0.081 | 10.401 | 0.163 |
| Reserves of pollen % dependent | 0.196 | 0.288 | 0.149 | 10.487 | 0.169 |
| Reserves of honey % dependent | 0.310 | 0.697 | 0.089 | 10.562 | 0.109 |

CE3, given sunflower exposure continued between CE2 and CE3, or at least to such differences being kept constant across treatments (CO, TH and CL), it did not happen that way. Moreover, between CE2 and CE3, PAB decreased at a greater proportion in CO than TH and CL groups. Other possible explanation is the fall in PAB due to a greater harvesting activity, at the same time derived from the better crop conditions when compared to those in CO. During harvesting, bees are most vulnerable, so greater foraging work implies a superior loss of foragers (Khoury et al., 2011). This would explain the case of TH, since honey and pollen increased, although this was not so clearly observed for those of CL.

On the other hand, based on Khoury et al. (2011), a significant effect should be considered when the difference is >7% in six days respect to control (EFSA, 2013). The difference exceeded that percentage, 8.1 and 8.8 points for TH and CL versus CO (Table 1 subparagraph C). However, these data correspond to seven days. A low effect can be assumed, when the reduction of PAB respect to control is between 7% and 15% (EFSA, 2013).

While PAB is a determining variable (EFSA, 2013), other variables provide relevant information regarding bee colonies status. Brood determines populations of later adult bees, so it is a fundamental factor for colony resilience. Dissenting results on brood have been obtained depending on test conditions (Pilling et al., 2013; Cutler et al., 2014; Sandrock et al., 2014; Rolke et al., 2016; Woodcock et al., 2017; Siede et al., 2018; Thompson et al., 2019). For the findings found, the same possible explanations for PAB would now serve. Thus, it would be easy to explain that the differences recorded in CE2 were caused by the treatments, but it would also contradict the fact that, in CE3 differences disappeared, although the exposition to sunflower remained. Flowering could provide a more solid explanation, since during an intense flowering at the end of the beekeeping season, bees direct efforts to foraging, which results detrimental for brood production. Unfortunately, there are insufficient data to support this finding.

The effect of neonicotinoids on reserves has been addressed in previous literature (Sandrock et al., 2014; Thompson et al., 2019). Pollen is an essential source for brood feeding, maintenance, and colony health (Brodschneider and Crailsheim, 2010; Mogren et al., 2019). Lack or low diversity of pollen weakens colonies, making them more susceptible to stress (Huang, 2012). Honey is the source of carbohydrates for bees. Bee hives exposed to TH showed higher accumulation than CO. Although, such difference was only significant in pollen in CE4, it may be attributed to crops status and environmental conditions found, more than to the bees themselves, given the relative homogeneity in the distribution of hives between the three treatments in each replicate. (Fig. 1; Table S2 and Table S6).

Hive weight is a common variable in the monitoring process of colonies, because it is an easily measureable variable. But, it does not discriminate the state of PAB, brood or reserves (Meikle and Holst, 2015; Flores et al., 2019). We report a lack of significant weight differences among treatments. However, colonies CO had significant more PAB and brood in CE2 and colonies TH significant more reserves in CE4. Therefore, we are in agreement with Khoury et al. (2013) and the weight only should be taken as an indicative information.

The control of Varroa was very effective during the first and third year. During the second year, hives reached blooms with a mite population greater than desired (Table S11). These bee hives were not treated in spring to avoid adding risk factors associated to the use of veterinary treatments. During periods of strong colony development, the parasite shows its greatest reproductive potential (Ronsenkranz et al., 2010). This probably caused the loss of some of the strongest colonies exposed to TH in replication 7, where 4 of the 6 colonies were lost (Table S12). Since no residues of thiamethoxam were detected in any of bee samples of this repetition, the high Varroa population could explain the colony loss. No symptoms of other diseases were detected, except for some isolated case of chalkbrood mummy.

Failures in queen and queen replacement is an important factor in colony loss (VanEngelsdorp et al., 2013). Sandrock et al. (2014) and Williams et al. (2015) found a significant effect on queens exposed to clothianidin and thiamethoxam, after artificially supplying pollen contaminated. Their findings shown that bee hives exposed to neonicotinoids significantly superseded more queens than the control. On the contrary, we could not detect significant difference among treatments (Kruskal-Wallis test, $p > 0.05$). Fifteen, eight and twelve queens were superseded in CO, TH and CL respectively. Seven, five and five of them happened during CE2 and CE3, and eight, three and seven from CE4 to CE6 (Fig. S2).

### 3.3. Colony mortality

Average mortality during three years was 26% (45 of 173 colonies). Mortality was distributed in 23.7% (14 colonies of 59), 28.1% (16 colonies of 57) and 26.3% (15 colonies of 57) for CO, TH and CL, respectively. No significant differences were detected among treatments (Kruskal-Wallis Test, $p > 0.05$). Colonies were grouped in quartiles, according to the initial population of bees in CE1. The proportion of dead colonies was higher among colonies that were weaker before being introduced into the blooms, with 34.9%, 30.2% 20.5% and 18.6% for 1 to 4 quartiles, respectively (Table S13; Table S12). Such mortality rate is considered within the normal mortality values observed by beekeepers in these regions and similar to those reported in literature (Potts et al., 2010; Woodcock et al., 2017; Thompson et al., 2019). Different factors may have caused colony loss, including replicate, as it can be inferred from the wide range of colony loss among replicates, ranging from 11.1% of replicate 8—43.8% of replicate 10 (Table S12); or bioclimatic conditions in each year, coinciding with some of the years with very harsh environmental conditions (Flores et al., 2019). On the other hand, data confirm the effect of the colony's strength on its survival. Thus, the tendency during the first year (Hernando et al., 2018) remained constant throughout the second and third year. There was a higher percentage of colony loss among bee hives with reduced strength in CE1 (distributed in quartiles, Table S13). Experimental conditions set to comply with EFSA's recommendations, such as the high isolation of the hives, or exposure to monocultures, in turn implies poor nutrition and probably contributed to higher mortality. The effect of the Varroa on bee hives exposed to TH in replicate 7 (Table S11), could be the cause for the loss of 4 hives that had displayed an excellent development up to that point. Concretely, the good development, and the previous failure to control of Varroa, enabled the mite to efficiently and successfully thrive and reproduce, hence when the control for Varroa was implemented, it was already too late to produce a significant improvement.

### 3.4. Residue analysis

Average recoveries were 79% and 85% (at 0.5 ng $g^{-1}$) for thiamethoxam and clothianidin in bee bread, within the 70—120% range. In adult bees, recovery was 89% and 82%, for thiamethoxam and clothianidin. RSD values were 8—16%. LC-QqQ-MS method yielded LOQ values of 0.5 ng $g^{-1}$ for clothianidin. For thiamethoxam, instrument detection limit (IDL) was 0.1 ng $g^{-1}$. Quantification of residues was calculated from the relative signal of each analyte in the sample extract versus the spiked extract from levels above LOQ, and within linearity range up to 300 ng $g^{-1}$. Any measurable concentration below LOQ is semi-quantitative, since

lower concentration levels were not fully evaluated for recovery and repeatability, so estimated uncertainty cannot be evaluated. For informative reasons, values ≤ LOQ are reported. In CE3, residues of thiamethoxam in bee bread were in the range of 0.10–0.37 ng. $g^{-1}$ (18 positives). In some colonies, residues of thiamethoxam were quantified or semi-quantified in 5 samples of adult bees in the range of 0.02–0.15 ng. $g^{-1}$, during the first year. Residues of clothianidin in bee bread were in the range of 0.1–2.89 ng. $g^{-1}$ (5 positives; in 11 samples, residues of clothianidin were semi-quantified from 0.1 to 0.48 ng. $g^{-1}$). Residues of clothianidin were semi-quantified in 4 samples of adult bees from 0.05 to 0.12 ng. $g^{-1}$. (Table S14). No neonicotinoid residues were measured in honey or brood. On the other hand, no residues were found in any of the samples in CE6. Seeds were analyzed and the dosage was checked (thiamethoxam, 0.25 mg a.s./seed; SD range 0.01–0.3) clothianidin, 0.5 mg a.s./seed; SD range 0.03–0.5). The toxic endpoints for acute oral exposure (LD50 = 0.005 μg/bee or 50 ng $g^{-1}$ and 0.0037 μg/bee or 37 ng $g^{-1}$) and chronic exposure (NOED = 10 days, 2 ng/bee and 10 μg.$L^{-1}$) for thiamethoxam and clothianidin, respectively, on adult honey bees (for individuals, and not at the colony level) represent a rough consensus of the toxicity studies reported in the literature (EFSA, 2013). The levels of residues detected are below those concentrations that produce toxic effects according to tests that consist of an individual level evaluation under laboratory conditions (OECD 213, 214 and 245, acute and chronic toxicity tests on *Apis mellifera L.*). As indicated above, this study does not evaluate effects at the individual level but at the colony level, as a superorganism.

## 4. Conclusions

The original raw data recorded in the bee hives do not allow us to establish a clear relationship between thiamethoxam and clothianidin and colony health, exposed to sunflower blooms, grown from seeds treated with these neonicotinoids. Using observations for each variable, relative to the initial status of colonies, there is a slight decrease in populations of bees and brood in colonies exposed to the treatments with respect to the control during the first week of exposure to sunflower blooms. Afterwards, the three groups of hives respond similarly, which could be due to several factors other than treatment, such as bloom quality. In addition, the lack of significant differences in the number of lost colonies in the three groups, the damage that Varroa produced in some bee hives of the second year, the lack of significant differences in the number of queen supersedures and traces of residues detected would support the hypothesis of a low effect due to treatments. Replicate and year were the factors that presented the greatest influence on the variability recorded in the different variables studied. On the contrary, the neonicotinoid treatments showed a null or very low effect on these variables. The low power of the effects of treatments can be the cause for the frequent contradictions that appear between results obtained at laboratory tests and those generated in realistic field conditions, at a large scale.

## Credit author statement

Victoria Gámiz, Sergio Gil and José M Flores (Departameno de Zoología. Universidad de Córdoba. Spain). They have worked in the design of the study, the development of all task to the honey bee management, bee hive assessment, sampling, evaluation of results and building of the article. Francisco Javier Navas (Departamento de Genética. Universidad de Córdoba). He is Statistical expert, and is responsible for all analysis of the data recorded during the three years. Inmaculada Rodríguez (Departamento de Bromatología. Universidad de Córdoba). She is expert on bee hives´ products. She has been the responsible and author of all melissopalinological analysis for confirm the exposition of bees to sunflower. Ana I García (Dpto. de Medio Ambiente y Agronomía, INIA). She is an chemistry analytical expert. She has been responsible for the analysis of neonicotinoids in treated seeds. Víctor Cutillas and Amadeo R. Fernandez-Alba (Dpto. de Física y Química. Universidad de Almería) and María Dolores Hernando Guil (Dpto. de Medio Ambiente y Agronomía, INIA). They have carried out the analysis and study of neonicotinoid residues in all samples of the study during the three years.

## Disclaimer

Responsibility for the information and views expressed in this manuscript lies entirely with the authors. The present manuscript is prepared under the sole responsibility of the authors.

## Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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## Appendix A. Supplementary data

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## References

Bate, S.T., Clark, R.A., 2014. The Design and Statistical Analysis of Animal Experiments. Cambridge University Press, Cambridge, UK.

Blacquière, T., Van der Steen, J.J., 2017. Three years of banning neonicotinoid insecticides based on sub-lethal effects: can we expect to see effects on bees? Pest Manag. Sci. 73, 1299–1304. https://doi.org/10.1002/ps.4583.

Brodschneider, R., Crailsheim, K., 2010. Nutrition and health in honey bees. Apidologie 41, 278–294.

Carreck, N.L., 2017. A beekeeper's perspective on the neonicotinoid ban. Pest Manag. Sci. 73, 1295–1298. https://doi.org/10.1002/ps.4489.

Carreck, N.L., Ratnieks, F.L.W., 2014. The dose makes the poison: have "field realistic" rates of exposure of bees to neonicotinoid insecticides been overestimated in laboratory studies? J. Apicult. Res. 53, 607–614. https://doi.org/10.3896/IBRA.1.53.5.08.

Cohen, J., 1988. Statistical Power Analysis for the Behavioral Sciences. Lawrence Earlbaum Associates. Inc, Hillsdale, NJ.

Crawley, M.J., 2012. The R Book. John Wiley & Sons, Hoboken, NJ.

Cresswell, J.E., Desneux, N., VanEngelsdorp, D., 2012. Dietary traces of neonicotinoid pesticides as a cause of population declines in honey bees: an evaluation by Hill's epidemiological criteria. Pest Manag. Sci. 68, 819–827.

Cutler, G.C., Scott-Dupree, C.D., Sultan, M., MacFarlane, A.D., Brewer, L., 2014. A large-scale field study examining effects of exposure to clothianidin seed-treated canola on honey bee colony health. development. and overwintering success. Peer J 2. 2, e652 https://doi.org/10.7717/peerj.652.

EFSA (European Food Safety Authority), 2013. Guidance Document on the risk assessment of plant protection products on bees (Apis mellifera. Bombus spp. and solitary bees). EFSA J. 11, 3295. https://doi.org/10.2903/j.efsa.2013.3295.

EFSA (European Food Safety Authority), 2018a. Conclusion on the peer review of the pesticide risk assessment for bees for the active substance clothianidin considering the uses as seed treatments and granules. EFSA J. 16 (2), 5177. https://doi.org/10.2903/j.efsa, 86 pp.

EFSA (European Food Safety Authority), 2018b. Conclusions on the peer review of the pesticide risk assessment for bees for the active substance thiamethoxam

considering the uses as seed treatments and granules. EFSA J. 16 (2), 5179. https://doi.org/10.2903/j.efsa.2018.5179, 59 pp.
Field, A.P., 2000. Discovering Statistics Using SPSS for Windows: Advanced Techniques for the Beginner. Sage Publications.
Flores, J.M., Gil-Lebrero, S., Gámiz, V., Rodríguez, M.I., Ortiz, M.A., Quiles, F.J., 2019. Effect of the climate change on honey bee colonies in a temperate Mediterranean zone assessed through remote hive weight monitoringsystem in conjunction with exhaustive colonies assessment. Sci. Total Environ. 653, 1111–1119.
Goulson, D., Nicholls, E., Botias, C., Rotheray, E.L., 2015. Bee declines driven by combined stress from parasites. pesticides. and lack of flowers. Science 347. https://doi.org/10.1126/science.1255957 (1255957–1255957).
Hakme, E., Lozano, A., Gómez-Ramos, M.M., Hernando, M.D., Fernández-Alba, A.R., 2017. Non-target evaluation of contaminants in honey bees and pollen samples by gas chromatography time-of-flight mass spectrometry. Chemosphere 184, 1310–1319.
Hernando, M.D., Gámiz, V., Gil-Lebrero, S., Rodríguez, I., García-Valcárcel, A.I., Cutillas, V., Flores, J.M., 2018. Viability of honey bee colonies exposed to sunflowers grown from seeds treated with the neonicotinoids thiamethoxam and clothianidin. Chemosphere 202, 609–617. https://doi.org/10.1016/j.chemosphere.2018.03.115.
Huang, Z., 2012. Pollen nutrition affects honey bee stress resistance. Terr. Arthropod Rev. 5, 175–189.
IBM Corp, 2016. IBM SPSS Statistics for Windows, Version 24.0. IBM Corp, Armonk, NY.
Johnson, R.M., Ellis, M.D., Mullin, C.A., Frazier, M., 2010. Pesticides and honey bee toxicity USA. Apidologie 41, 312–331. https://doi.org/10.1051/apido/2010018.
Khoury, D.S., Barron, A.B., Myerscough, M.R., 2013. Modelling food and population dynamics in honey bee colonies. PloS One 8, e59084. https://doi.org/10.1371/journal.pone.0059084.
Khoury, D.S., Myerscough, M.R., Barron, A.B., 2011. A quantitative model of honey bee colony population dynamics. PloS One 6, e18491. https://doi.org/10.1371/journal.pone.0018491.
Lundin, O., Rundlöf, M., Smith, H.G., Fries, I., Bommarco, R., 2015. Neonicotinoid insecticides and their impacts on bees: a systematic review of research approaches and identification of knowledge gaps. PloS One 10, e0136928. https://doi.org/10.1371/journal.pone.0136928.
Meier, U., 2001. Growth stages of mono-and dicotyledonous plants – BBCH Monograph. The BBCH codes are on: http://www.reterurale.it/downloads/BBCH_engl_2001.pdf.
Meikle, W.G., Holst, N., 2015. Application of continuous monitoring of honey bee colonies. Apidologie 46, 10–22.
Mogren, C.L., Danka, R.G., Healy, K.B., 2019. Larval pollen stress increases adult susceptibility to clothianidin in honey bees. Insects 10. https://www.mdpi.com/2075-4450/10/1/21/pdf.
Odemer, R., Nilles, L., Linder, N., Rosenkranz, P., 2018. Sublethal effects of clothianidin and Nosema spp. on the longevity and foraging activity of free flying honey bees. Ecotoxicology 27, 527–538. https://doi.org/10.1007/s10646-018-1925-5.
OECD Test No. 213. Honey bees. Acute oral toxicity test. https://www.oecd-ilibrary.org/environment/test-no-213-honey bees-acute-oral-toxicity-test_9789264070165-en.
OECD Test No. 214. Honey bees. Acute contact toxicity test. https://www.oecd-ilibrary.org/environment/test-no-214-honey bees-acute-contact-toxicity-test_9789264070189-en.
OECD Test No. 245. Honey bee (Apis mellifera L.). Chronic oral toxicity test (10-day feeding). https://www.oecd.org/env/ehs/testing/test-no-245-honey-bee-apis-mellifera-l-chronic-oral-toxicity-test-10-day-feeding-9789264284081-en.htm.
Pilling, E., Campbell, P., Coulson, M., Ruddle, N., Tornier, I., 2013. A four-year field program investigating long-term effects of repeated exposure of honey bee colonies to flowering crops treated with thiamethoxam. PloS One 8, e77193. https://doi.org/10.1371/journal.pone.0077193.
Potts, S.G., Biesmeijer, J.C., Kremen, C., Neumann, P., Schweiger, O., Kunin, W.E., 2010. Global pollinator declines: trends. impacts and drivers. Trends Ecol. Evol. 25, 345–353.
Rolke, D., Fuchs, S., Grünewald, B., Gao, Z., Blenau, W., 2016. Large-scale monitoring of effects of clothianidin-dressed oilseed rape seeds on pollinating insects in Northern Germany: effects on honey bees (*Apis mellifera*). Ecotoxicology 25, 1648–1665.
Richardson, J.T., 2011. Eta squared and partial eta squared as measures of effect size in educational research. Educ. Res Rev. Neth. 6, 135–147.
Rosenkranz, P., Aumeier, P., Ziegelmann, B., 2010. Biology and control of Varroa destructor. J. Invertebr. Pathol. Suppl 1, 96–119. https://doi.org/10.1016/j.jip.2009.07.016. Epub 2009 Nov 11.
Sánchez-Bayo, F., 2014. The trouble with neonicotinoids. Chronic exposure to widely used insecticides kills bees and many other invertebrates. Science 346, 806–807.
Sandrock, C., Tanadini, M., Tanadini, L.G., Fauser-Misslin, A., Potts, S.G., Neumann, P., 2014. Impact of chronic neonicotinoid exposure on honey bee colony performance and queen supersedure. PloS One 9, e103592. https://doi.org/10.1371/journal.pone.0103592.
Schmuck, R., Lewis, G., 2016. Review offield and monitoring studies investigating the role of nitro-substituted neonicotinoid insecticides in the reported losses ofhoney bee colonies (Apis mellifera). Ecotoxicology 25, 1617–1629. https://doi.org/10.1007/s10646-016-1734-7.
Siede, R., Meixner, M., Almanza, M.T., Schoning, R., Maus, C., 2018. A long-termfield study on the effects of dietary exposure of clothianidin to varroosis-weakened honey bee colonies. Ecotoxicology 27, 772–783.
Schmoldt, D., 1993. Assessing the Effects of Fire Disturbance on Eco-Systems, vol. 104. Diane Publishing Company.
Terrab, A., Díez, M.J., Heredia, F.J., 2003. Palynological, physico-chemical and colour characterization of Moroccan honeys: III. Other unifloral honey types. I. J. Food Sci. Technol. 38, 395–402.
Thompson, H., Overmyer, J., Feken, M., Ruddle, N., Vaughan, S., Scorgie, E., Bocksch, S., Hill, M., 2019. Thiamethoxam: long-term effects following honey bee colony-level exposure and implications for risk assessment. Sci. Total Environ. 654, 60–71.
VanEngelsdorp, D., Tarpy, D.R., Lengerich, E.J., Pettis, J.S., 2013. Idiopathic brood disease syndrome and queen events as precursors of colony mortality in migratory beekeeping operations in the eastern United States. Prev. Vet. Med. 108, 225–233.
Vázquez, P.P., Lozano, A., Uclés, S., Ramos, M.M.G., Fernández-Alba, A.R., 2015. A sensitive and efficient method for routine pesticide multiresidue analysis in bee pollen samples using gas and liquid chromatography coupled to tandem mass spectrometry. J. Chromatogr. A 1426, 161–173.
Williams, G.R., Troxler, A., Retschnig, G., Roth, K., Yanez, O., Shutler, D., Neumann, P., Gauthier, L., 2015. Neonicotinoid pesticides severely affect honey bee queens. Nat. Sci. Rep. 5, 14621. https://doi.org/10.1038/srep14621.
Wood, S.C., Kozii, I.V., Medici de Mattos, I., Carvalho Macedo Silva, R., Klein, C.D., Dvylyuk, I., Moshynskyy, I., Epp, T., Simko, E., 2020. Chronic high-dose neonicotinoid exposure decreases overwinter survival of Apis mellifera L. Insects 11, 30. https://doi.org/10.3390/insects11010030.
Woodcock, B.A., Bullock, J.M., Shore, R.F., Heard, M.S., Pereira, M.G., Redhead, J., Ridding, L., Dean, H., Sleep, D., Henrys, P., Peyton, J., Hulmes, S., Hulmes, L., Sárospataki, M., Saure, C., Edwards, M., Genersch, E., Knäbe, S., Pywell, R.F., 2017. Country-specific effects of neonicotinoid pesticides on honey bees and wild bees. Science 356, 1393–1395.