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Viability of honeybee colonies exposed to sunflowers grown from seeds treated with the neonicotinoids thiamethoxam and clothianidin.

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Abstract

In this study, honeybee colonies were monitored in a field study conducted on sunflowers grown from seeds treated with the systemic neonicotinoids thiamethoxam or clothianidin. This field trial was carried out in different representative growing areas in Spain over a beekeeping season. The health and development of the colonies was assessed by measuring factors that have a significant influence on their strength and overwintering ability. The parameters assessed were: colony strength (adult bees), brood development, amount of pollen and honey stores and presence and status of the queen. The concentration of residues (clothianidin and thiamethoxam) in samples of beebread and in adult bees was at the level of $ng.g^{-1}$; in the ranges of 0.10 – 2.89 $ng.g^{-1}$ and 0.05 – 0.12 $ng.g^{-1}$; 0.10 – 0.37 $ng.g^{-1}$ and 0.01 – 0.05 $ng.g^{-1}$, respectively. Multivariate models were applied to evaluate the interaction among factors. No significant differences were found between the honeybee colonies of the different treatment groups, either exposed or not to the neonicotinoids. The seasonal development of the colonies was affected by the environmental conditions which, together with the initial strength of the bee colonies and the characteristics of the plots, had a significant effect on the different variables studied.

Keywords: neonicotinoid residues, honeybee colony, higher tier study, sunflower crop

INTRODUCTION

In recent years, severe losses in honeybee colonies have occurred largely due to health problems linked to parasites such as the *Varroa destructor* mite and other pathogens. In addition, toxic effects due to a direct or indirect exposure of bees to some veterinary treatments for controlling bee diseases, as well as other chemicals as plant protection products (PPPs) used in farming, are also considered [Chauzat et al., 2007; Potts et al., 2010; Serra-Bonvehí et al., 2010; Smith et al., 2013; Boi et al., 2015]. This latter factor has been under debate in recent times, and the EU has restricted the use of some agricultural treatments, specifically three neonicotinoid substances [Regulation EU No 485/2013]. This has led to divergent conclusions among chemical companies, farmers, beekeepers, researchers and environmentalist organisations [Johnson et al., 2010; Creswell et al., 2012; Sánchez-Bayo et al., 2014; Sandrock et al., 2014; Lundin et al., 2015; Blacquière et al., 2017; Carreck, 2017]. Since keeping honeybee colonies healthy is critical for the survival of the species itself, for beekeepers and for the pollination of many plants and essential crops for human nourishment, there is a focus on the evaluation of toxicological risks of the substances used in agriculture.

In this regard, ecotoxicological studies of plant protection products (PPPs) are required before they authorised and released on the market [Regulation 1107/2009] in order to demonstrate that following good agricultural practices, they will not cause any risk on populations of non-target organisms in the field. Evaluating PPPs involve laboratory- and field-based studies for characterising hazards and risks, which entails strengths and weaknesses of both approaches. In field studies, there is an inherent variability and they often employ a large sample size to associate effects with treatments, what makes them expensive and unfortunately, less common [Lundin et al., 2015]. Such type of studies have been carried out mainly on rapeseed or maize [Pilling et al., 2013; Cutler et al.,

2014; Lundin et al., 2015; Rolke et al., 2016; Schmuck et al., 2016; Woodcock et al., 2017]. While laboratory-based research can provide a finding with a high level of precision, it is more unlikely to reflect a real scenario. To evaluate or estimate the effects of neonicotinoids on individual bees, several studies have been conducted and partly, controversially interpreted [Johnson et al., 2010; Carreck et al., 2014; Sánchez-Bayo et al., 2014; Sandrock et al., 2014; Lundin et al., 2015; Pisa et al., 2015; Blacquière et al., 2017]. However, the potential impact of neonicotinoids to bees is still not fully characterised. It is well recognized that the effects of neonicotinoids should be assessed not only on individual bees, but also on colony level. All this still reflects the need for the further development of concepts and methodologies for assessing the health status of the entire colony, and whether this superorganism can compensate for stress factors.

The work presented here is a large-scale field study, an approach to improve knowledge about exposure of honeybees on sunflowers grown from seeds treated with the systemic neonicotinoids thiamethoxam and clothianidin, two of the substances restricted for their use as systemic seed treatments for sunflowers in the EU. Sunflower is a common crop in various regions of Europe [Eurostat, 2017]; and it has flowering period which is late in the beekeeping season. The development and health status of the exposed colonies was assessed by measuring factors that have an influence to the strength and overwintering ability: colony strength (number of adult bees), brood development, stores of pollen and honey, presence and status of the queen and bee disease symptoms. The study has been carried out in four regions of Spain; Andalusia, Castilla-La Mancha, Madrid and Extremadura.

2. EXPERIMENTAL

2.1. Study locations

The four selected locations (or replications) are located on rainfed land in central and southern Spain. In each replication, three cultivation plots were selected, one for each treatment (T) (***Fig S1*** *supporting information)*. To tackle the use of commercial fields for experimental studies of PPPs under restriction by EU regulation, it was required approval by the Competent Administration Authority. Sunflower crop (*Helianthus annuus*) grown from (i) untreated seeds – T1, (ii) seeds treated with thiamethoxam (Syngenta's product Cruiser® FS 350 g/L, at a rate of 0.25 mg a.s./seed)- T2, and (iii) seeds treated with clothianidin (Bayer's product Poncho® FS 600 g/L, at a rate of 0.5 mg a.s./seed)- T3. In order to obtain good development in the plants, seeds from each of the three treatment groups were also treated with metalaxyl and fludioxonil (Syngenta's fungicides Apron® XL M ES 339 g/L, at a rate of 0.3 L/100 kg seed, and Celest® Formula M FS 250 g/L, at a rate of 0.5 L /100 kg seed). No other treatment was applied afterwards on the experimental plots. Sunflower seeds of the hybrid variety Bosfora® (Syngenta) were used. The seeds were treated according to commercial seed treatment methods at the rates referred to above (at the Bayer Seed Growth Center, Monheim, Germany). Two-hectare plots were planted, separated from each other by at least 2 Km when possible (***Table S1***). The colonies were placed at the center of each plot. The plots were randomly assigned to each treatment, except when the distance between crops could not be greater than two kilometres. In such cases, the control plot was the most distant from the treated plots.

2.2. Honey bee colony handling and management

In each replication the three treatment groups were evaluated, and for each treatment and replication, six hives were used, resulting in a total of 72 hives assessed (***Fig S1)***.

Seventy two swarms of honey bees (*Apis mellifera iberiensis*, Engel) were obtained from professional beekeepers (it is a western honey bee subspecie native to the Iberian Peninsula, from Sierra Morena, Córdoba, managed following the traditional methods). The bee swarms were housed in Langstroth hives, provided with screening bottom boards to detect and control *Varroa destructor* [Flores et al., 2000; Dietemann et al., 2013]. All swarms were requeened with queens reared in early spring, with free mating (queens were reared at the experimental apiary, University of Córdoba). All queens were marked. Each week, the colonies were fed, 1L of sugar syrup (50% water / 50% sucrose) with the addition of 10 $mL.L^{-1}$ of nutritional supplement for bees (Promotor L47®; Laboratories Calier) for five weeks to promote colony growth. During the colonies' development period, comb foundations of conventional wax were supplied. Natural pollen harvesting was sufficient in this place and in this period. The hives were managed as in standard professional beekeeping.

The beehives were placed on wooden supports and were painted with different colours to reduce drifting of the bees between the different hives. The beehives were located in a Mediterranean forest area isolated from other crops (Santa María de Trassierra, Córdoba, Spain), except during sunflower bloom. The colonies were provided with water sources in nearby areas at all the time: a natural creek in the forest location and watering holes for bees during the sunflower flowering period.

2.3. Assessment of the honeybee colonies

At the beginning of the study, each beehive was weighed using a crane scale (GRAM CR-150®) installed on a metal lever. For colony assessments, the following parameters were recorded: colony strength (number of adult bees per hive), comb surface covered by brood, comb surface covered with pollen and honey stores, presence and status of the

queen and symptoms of bee diseases. To determine the colony strength, each colony was opened and all combs were sequentially removed and weighed with the adult bees on a digital scale (EKS. SWEET 8231 SW**)**. Estimation of colony size was carried out using digital photography. Once weighed, the frames were placed in a box compatible with Langstroth model frames. When all combs were removed, the number of adult bees remaining on the walls of the hive was estimated. Liebefeld method was only used for estimation the number of bees at the bottom of the hive [Imdorf and Gerig, 2001]. Next, adult bees from each frame were brushed into the hive and the combs were weighed again without bees. A sample of bees was weighed and counted to obtain the average weight per bee (approx. 10g or 100 bees). Then, the total number of bees was calculated based on the total weight of the bees in the beehive. A picture of both sides of each frame without bees (after brushing them into the hive) was taken (Nikon reflex camera D5100; 18/55VR lens). The photographs were later processed with image analysis software (Image J®) in order to determine the area in cm^2 occupied by honey, pollen, open and sealed worker brood and drone brood. The presence of the original queen was checked in each colony. Once the queen was spotted, it was caged into a plastic queen cage clip bee catcher to prevent damages during handling.

During managing, the colonies were visually checked to detect any symptoms of disease, such as scattered brood, dead or damaged larvae or pupae, chalkbrood mummies or adult bee diseases were sought and recorded. The mite *Varroa destructor* continues to be considered the most devastating parasite of honeybee colonies. In temperate areas, an infested colony might dead unless there is intervention to reduce the mite population. In this regards, the application of veterinary treatments is mandatory in the Spanish regulation (Royal Decree 608/2006). The following measures to control *Varroa* were applied: two treatments against the parasite with Amicel® (i.e.

159 amitraz, 500 mg) applied in May and October 2015. The first treatment was required
160 because an infestation with the parasite was detected in some colonies. The second
161 treatment was preventive and prior to overwintering. The diagnosis of mite infestation
162 was conducted on three occasions: October 2015 (prior to treatment), December 2015
163 and March 2016, to determine the mite fall over four days [Flores et al., 2002]. No other
164 disease symptoms were observed in any of the beehives for the entire duration of the
165 study. The parasite Varroa was controlled at all times during the study.

166 Each colony was assessed six times. The first assessment was carried out just before the
167 colonies were moved to the study plots at sunflower bloom (A1). It was the starting
168 point to determine the colony status in which the beehives were introduced into the
169 flowering sunflower plots. This examination was also used as the basis for distribution
170 of the beehives at each replication between the three treatment groups as
171 homogeneously as possible. Then, each group of hives was randomly assigned to a
172 treatment. In order to ensure maximum exposure of the bees to sunflowers blooms and
173 prevent dilution of the nectar and pollen collected by bees with the previously existing
174 nectar stores in the hives, all the combs with no brood were removed and replaced with
175 combs from organic beekeeping. The beehives were placed in the sunflower crops at the
176 beginning of blooming, when at least 20% of the plants were at the phenological growth
177 stage 61-62 of the BBCH scale (Biologische Bundesanstalt, Bundessortenamt and
178 Chemical Industry) [Meier, 2001]. The second assessment (A2) took place a week after
179 introduction of the beehives into the sunflower crops (***Fig S2***). The third one (A3), three
180 weeks after, when the flowering sunflowers was in the phenological state 75-79 of the
181 BBCH scale. A2 and A3 were carried out during the period when colonies were
182 exposed to the crop (three weeks) and they were considered an evaluation of potential
183 short-term effects. The origin of the analysed honey samples regarding floral sources

184 (collected in A3) was determined according to the methods established by The
185 International Commission for Bee Botany [Louveaux, 1978, Erdtman, 1960]. The
186 pollen count was conducted using an optical microscope. In the case of sunflower
187 honey, since there are sunflower varieties that produce low amounts of pollen, the
188 general criterion to classify honey as monofloral from *Helianthus annuus* was a
189 percentage of 15 or 20 % sunflower pollen [Accorti et al., 1986; Pérez et al., 1994;
190 Mateo et al., 1998; Terrabet al., 2003, Nicolson et al., 2013]. A percentage > 23% of
191 sunflower pollen was used for classifying. A4 was carried out at the end of summer; this
192 is an extremely unfavourable season for bees in southern Spain, with high temperatures
193 (up to 40ºC; AEMET, 2015), no precipitation and little natural food sources available.
194 For this reason, each beehive was supplied with food ad libitum (23.6 % saccharose,
195 23.6 % dextrose, 52.8% glucose syrup) for maintenance throughout the summer
196 (approx. 2kg per hive). A5 was carried out in December, during early winter.
197 Assessments A4 and A5 were considered for the evaluation of potential mid-term
198 effects. In southern Spain, temperatures remain moderate during this period and some
199 flowers can still be in bloom due to autumn rain [AEMET, 2011]. This allows for bee
200 colonies to keep a certain amount of brood throughout the winter. The sixth assessment
201 (A6) was carried out in early March 2016. This assessment was considered to provide
202 results regarding potential long-term effects, since it was possible with this assessment
203 to record the overwintering success and the colonies' response at the start of the new
204 beekeeping season (***Fig S2***).

205 Samples of adult bees (approx. 10g, collected from the bottom of the beehive,
206 presumably with a higher proportion of foragers) and a piece of combs with beebread
207 (approx. 25 cm^2) of each colony were collected for residue analysis. All the samples

were labelled accordingly and quickly placed in coolers with ice sheets, then stored at -20°C once back in the laboratory to be analysed.

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 of residues were based on LC-Q-Orbitrap-MS. An UHPLC Dionex™ Ultimate 3000 (Thermo ScientificTM, San Jose, USA). A QExactive Focus (Thermo Scientific, Bremen, Germany) mass spectrometer was equipped with Heated Electrospray Ionization Source (HESI II). Samples of beebread and adult bees collected after three weeks of exposure (A3) were analyzed. The extraction procedures of neonicotinoid residues from samples of adult honeybees and beebread (isolated from the comb), and acquisition parameters for residue analysis by LC-QqQ-MS and LC-Q-Orbitrap-MS are described in supplementary material based on previous publications [Hakme et al, 2017; Vázquez et al., 2015].

Method validation and performance for quantification of neonicotinoid residues were carried out in accordance with quality control standards [ISO 17025]. Identification of residues in samples of beebread and in adult bees, was based on: acquisition of 2 SRM (selected reaction monitoring) transitions, retention time (tolerance of ±0.2 min) and SRM ratio compliance (a relationship between abundance of selected SRM transitions for identification and quantification, SRM_2/SRM_1 with a tolerance of ±30%). For quantitative analysis of residues, we aimed to use standard addition involving measurement of the responses of the analyte in a sample extract versus spiked sample

231 extract, because this approach has the potential to determine ion suppression or
232 enhancement, as result of a matrix as complex as beebread or adult bees.
233
234 **2.5 Data analysis**
235 The results were statistically processed using SPSS software for Windows 17.0®.
236 Parametric statistics were applied when possible. When, the distribution was unknown
237 due to a small sample size, or there was no variance homogeneity, non-parametric
238 statistics were used (test of Kolmogorov-Smirnov). Multivariate models were applied to
239 evaluate the interaction among factors. The Pearson Correlation test was used to study
240 the interactions among the variables recorded in the colonies (see supplementary
241 material).
242

243 **3. RESULTS AND DISCUSSION**

244 In order to evaluate the potential effects of neonicotinoid residues, the sunflower plots
245 were selected in a way that they were as isolated as possible from other entomophilous
246 crops in bloom, from other plant species that could be attractive to bees, and from the
247 different treatment groups of each replication. The field study was designed considering
248 the risk framework for PPPs (EPPO 170a, EFSA 2013). The analysis of the origin of the
249 honey proved the exposure to sunflower crops (the shares of sunflower pollen in the
250 honey samples were in the range of 23.3-60.4%). The hot, the dry weather conditions,
251 and low availability of other floral resources for bees during summer, when sunflowers
252 commonly bloom, made a particularly high degree of isolation necessary. These
253 conditions and the removal of food stores before placing the beehives in the study
254 flowering fields have allowed to test a worst-case scenario in this study. The data

obtained to assess the status of the colonies have been generated in the temperate/warm climate of southern Europe.

3.1. Development of colony strength

The results show that the initial number of adult bees had a strong influence on the development of this parameter throughout the study until the end. When considering bee colonies exposed to blooming sunflower fields grown from seeds treated with neonicotinoids (T2 and T3), it can be seen that the development of colony strengths follows the same pattern as in the control group (T1). Based on the model by Khoury et al. (2011), it is predicted that a reduction of colony strength of lower than 7% in 6 days compared to the control colonies can be considered to be a negligible effect [EFSA Guidance Document, 2013]. For T2 (thiamethoxam), the decline in colony strength observed during the first week of exposure to flowering was almost the same as the one in T1 (3.76 % and 3.44 %, respectively). This decline was slightly greater for T3 (clothianidin) by 4.16 % (7.60% - 3.44%, respectively), **Table 1a**. In a second stage of the evaluation (at the *mid and long-term*), the decrease of colony strength was balanced out after 7 days of exposure [Khoury et al., 2011; EFSA Guidance Document, 2013]. The colony strengths, from day 7 to 21 of exposure were lower in T2 and T3 compared to T1, however, without statistically significant differences (one-way ANOVA $p<0.05$). Similar developments were observed two months later, and during the recovery period of bees after the harsh conditions in summer, in autumn (A5) and early spring (A6). The development of the average numbers of adult bees per hive in the different treatment groups was very similar and in line with the development of beehive weight registered. No significant differences were observed among treatments for any of the six evaluations (one-way ANOVA $p<0.05$, ***Table S2***). There was a sharp decline in colony strengths in all treatment groups from the moment the beehives were introduced into the

blooming sunflower fields until the autumn (A5). To get a better understanding of the natural dynamics of colony strengths under the conditions of our study, we focused on the control colonies that survived until the end of the study (n = 20 colonies). In T1, the colony strengths fell by 3.44% in the first week the colonies spent in the blooming sunflower fields, by 18.29% over the whole time spent in the blooming sunflower fields, and by 51.09% until A4 (September), in the last period of summer. These colonies were able to recover afterwards, when the new supply of natural food reactivated the colonies. The initial reduction could be explained by the potential increase in the number of foraging bees, presumably due a precocious transition from nurse bees to foragers, as they were introduced into the flowering fields, which in turn would lead to a decline in the average life span of the total hive population [Khoury et al., 2011 and 2013]. This would be aggravated by the previous removal of a large portion of the food stores, which would demand a greater foraging effort from the bees [Schulz, 1998]. The decline of colony strengths recorded at the end of the flowering period could be explained by the same reasons, in addition to the significant reduction of brood (future adult bees) during the flowering period, which probably had the effect of intensifying the decline of the colony strengths. All of these facts continued to have an impact during the summer, adding to the fact that there were no longer any flowering plants that could be used by the bees for foraging. It should be noted that this is the situation that a large portion of bee colonies face in the southern part of Spain, and probably in other southern regions of Europe as well. Within Europe there are variations in the models of colony development as a result of adaptation to different climate zones [Hatjina et al., 2014], and what it is observed in this study could be interpreted as a mechanism to adapt to local conditions. It is possible that the evolution of colonies would have been different if after the sunflower blooming period, the beehives had been

relocated somewhere with more favourable climatic conditions and flowering fields available, which would have helped them to recover faster. However, this study was designed as a worst-case scenario that ensured maximum exposure of the bees to T2 and T3 and assessment of the bee health status at mid to long term.

3.2. Colony losses

The number of colonies that died throughout the study was 4, 6, and 7 for T1 (control), T2 and T3, respectively, **Table 1b and 1c.** No statistically significant differences between the treatment groups were observed (Non-parametric statistics, Kruskal-Wallis test, p=0.639). The initial colony strength was a significant factor to explain the mortality of the colonies, since the highest number of dead colonies was recorded among the beehives that were initially the weakest. Therefore, colonies were grouped in quartiles, taking as a benchmark the number of adult bees, with higher mortality in the first two quartiles. This is important since both T2 and T3 initially had a higher number of beehives from quartiles 1 and 2 than T1 (15, 13 and 8 respectively). Therefore, the weakness of the colonies is a key factor affecting their mortality, regardless of the treatments. Indeed, the weaker beehives (designed as quartile 1 and 2) were also the ones most affected, since a greater number of combs containing food stores were removed. On the other hand, in a large number of colonies there were problems with the queens (***Table S3***), since a big proportion of the dead beehives replaced queens during the period of high temperatures, during the sunflower flowering period and summer. On other occasions, the queens had problems to keep up adequate egg-laying. These situations occurred in all treatments. In this study, for all quartiles, the development of colony strengths followed the same pattern (***Table S4***). No statistically significant differences among treatments were observed (Non-parametric statistics, Kruskal-Wallis test, p=0.639).

3.3. Status of queen

At the end of the study, the same number of queens had been replaced in all treatment groups except for T2, where one colony less had the queen replaced. It should be noted that before the evaluation A1, two, four and five bee colonies already replaced their queens for T1, T2 and T3, respectively. At that time the bees had not yet been in contact with the sunflower fields. In A2 and A3, with the beehives set up in the sunflower fields, it was observed that T1 and T2 had the largest number of beehives with their queens replaced (three beehives in each treatment) than T3 (only one beehive) (***Fig S3***). Queen replacement is very common among honeybee colonies normally used in southern Spain, where these trials were carried out (based on personal observation and practical experience of professional beekeepers). Recently, high temperatures have been considered as a reason for the failure of queens [VanEngelsdorp et al., 2013; Pettis et al., 2016], a circumstance that occurred in these studies.

3.4. Development of brood

The total comb surface covered with worker brood was considered to be the sum of all cells containing eggs and open and sealed worker bee brood. Likewise, the area occupied by drone brood was also registered. In both cases, an overall development was observed in all colonies that follows the same pattern registered for colony strengths: a progressive decrease of brood started when the beehives were placed in the crop, which does not change its trend until the beginning of the autumn. The dynamics of brood development was similar for all treatments (***Fig S4***) and no statistically significant differences were observed in the brood area between the treatments, neither for worker nor for drone brood (one-way ANOVA $p<0.05$), except for A3 of the second replication, in which T1 (control) had a greater amount of drone brood than T2 (Kruskal-Wallis non-parametric test $p<0.05$, Mann-Whitney U test $p<0.05$, ***Table S5***).

The decline in brood production explains part of the subsequent decline in colony strengths observed in the study. This situation is a common reaction of bee colonies in temperate climates with periods of high temperatures and scarce precipitation like the one in which these trials have been carried out, which is different from the conditions observed in other locations in Europe, where the fall in populations occurs mainly before overwintering. In the conditions of southern Spain, an "oversummering" should probably be considered as well as an overwintering, as proven by the fact that the higher percentage of dead beehives actually occurred before winter (**Table 1a**). It should be noted that the amount of brood in a honeybee colony is part of its strength, which must also be related to adult bee population and food stores. Brood is heavily impacted by climate and food conditions. It is affected by climatic conditions in various ways: the climate itself, which influences the food supply, or high temperatures and low humidity, which also have a negative impact on brood production in honey bee colonies [Doull, 1976]. It should also be taken into account that sunflower pollen is considered to be of low quality because of its protein content and amino acid composition [Collison, 2016]. This is important for understanding the health status of the beehives and of the development of colony strengths, since the two variables are correlated (**Table 2**).

3.5. Pollen and honey stores

Beehives were placed into flowering sunflower fields with few food stores in the hives, since all frames with pollen and honey without brood had previously been removed at evaluation A1. A slight increase of pollen and honey stores was observed during the week after the beehives were placed in the sunflower fields, and registered during A2. This increase of honey stores continued in A3 for T2 and T3, but not for the control. (***Table S6***). However, pollen stores decreased (***Table S7***), because all the food collected was consumed by the adult bees and bee brood, in addition to building new combs to

replace the removed ones. In summer, the average sizes of food stores fell because of the low availability of natural food sources, and the bees had to use their reserves to survive until the autumn. This probably also had an effect on colony strength and brood. When the colonies were removed from the blooming sunflower fields, they were supplied with artificial diet ad libitum due to the alarming lack of honey reserves, which would have caused a collapse of many of the colonies due to starvation over the summer. This was emergency feeding intended for immediate consumption, and bees rarely stored it in the comb cells. For this reason, in A4 the food stores were still declining in size. During autumn, there was a new increase in stored honey that remained until the beginning of spring (A6). No significant differences were detected between treatments for pollen or honey stores in any of the assessments (one-way ANOVA $p<0.05$).

3.6. Beehive weight

No significant differences were observed between treatments in any of the evaluations (one-way ANOVA $p<0.05$). It is not unusual that bee hives gain weight rather slowly during early spring because, although the colonies are growing, the storage of honey is low. And afterwards it may increase its weight faster due to the increase of honey reserves, which is heavier, even though the bee colony strengths may be declining during that period. This is reflected in our data as well (***Table S8***), when comparing the weight of beehives to the number of bees per colony (***Fig S5 and S6***). The weight evolved as expected under study conditions, with a strong reduction from the sunflower flowering period to the beginning of autumn. It shows an evolution completely different to the one occurring in other parts of Europe, where it is actually in the summer when beehives store the highest amount of honey and bee hives are heavier [Lecocq et al., 2015].

3.7. Residue analysis

Identification was done for the selected compounds based on the monitoring of 2 SRM transitions using LC-QqQ-MS methodology and in accordance with quality control standards [ISO 17025]. When the identification was especially problematic, LC-Q-Orbitrap-MS methodology was applied to obtain a high-resolution of both full-scan and fragmentation mass spectra. The method‘s LOQ (limit of quantification), using LC-QqQ-MS methodology, is defined as the minimum concentration that can be quantified with acceptable accuracy and precision. Acceptable mean recoveries were those within the 70-120 % range and, with an associated precision of RSD < 20%. Average recoveries were 79 % and 85% (at 0.5 $ng.g^{-1}$) for thiamethoxam and clothianidin in beebread. In adult bees, recovery results were 89% and 82%, at 0.5 $ng.g^{-1}$, for thiamethoxam and clothianidin. Most of the RSD values were around 8-16%. LC-QqQ-MS based method yielded LOQ values of 0.5 $ng.g^{-1}$ for clothianidin. For thiamethoxam, the instrument detection limit (IDL) was 0.1 $ng.g^{-1}$ (with 2 SRM transitions, retention time (tolerance of ±0.2 min) and SRM ratio compliance). Quantification of neonicotinoid residues in samples was calculated from the relative responses of each analyte in the sample extract versus the spiked samples extracts from levels above LOQ, and within the instrumental linearity range up to 300 $ng.g^{-1}$. Any measurable concentration below LOQ can only be considered as semi-quantitative, since lower concentration levels were not fully evaluated for recovery and repeatability and therefore the estimated uncertainty cannot be evaluated. For informative reasons, values equal or below LOQ are reported. The concentration of residues of thiamethoxam in beebread was in the range of 0.10 – 0.37 ng. g^{-1} (13 positives among the total nº of colonies exposed to the treated sunflowers, which were 24). In some of these colonies, residues of thiamethoxam were semi-quantified in 5 samples of adult bees in the range

of 0.01-0.05 ng. g^{-1}. The concentration of residues of clothianidin in beebread was in the range of 0.10 – 2.89 ng. g^{-1} (5 positives among the total nº of colonies exposed to treated sunflowers, which were 24; in 8 samples, residues of clothianidin were semi-quantified in the range of 0.1 – 0.48 ng. g^{-1}). Residues of clothianidin were semi-quantified in 3 samples of adult bees in the range of 0.05-0.12 ng. g^{-1}. In 4 beehives; of which 2 correspond to colonies that not survived and 2 others that survived through the beekeeping season, residues were quantified or semi-quantified in samples of beebread and adult bees, collected from the same beehive (**Table 3**). Thiamethoxam was determined at 0.37 and 0.02 ng.g^{-1} in beebread and adult bee, respectively; clothianidin at 2.89 and 0.07 ng.g^{-1}, respectively. In the 2 colonies that survived, thiamethoxam was determined at 0.22 and 0.05 ng.g-1, in beebread and adult bee, respectively; and clothianidin, at 2.58 and 0.12 ng.g^{-1}, respectively. No neonicotinoid residues were measured in other colonies that did not survive (**Table 3**). The toxic endpoints for acute oral exposure (LD_{50} = 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 (individuals) represent a rough consensus of the toxicity studies reported in the literature, [EFSA, 2013]. In the 2 failed colonies, the concentration of residues was below 3 ng.g^{-1}. As mentioned before, the effects of neonicotinoids need to be assessed not only on individuals, but also at the colony level. In this field study, no significant differences between control plots and treatments were observed. ***Table 3S*** shows potential causes of death for the colonies that did not survive.

3.8. Interactions among factors

Both location and beehive condition had a clear effect on all the parameters studied in the beehives. The treatment and the interaction of the treatment with the other two

455 factors only had a significant effect under exceptional circumstances (**Table 3).** All the
456 variables registered (weight, colony strength, brood, stored pollen and honey) are
457 significantly correlated to some extent (**Table 2**). Both the colony strength and brood
458 were strongly correlated with each other, also depending on environmental conditions,
459 especially on the amount of nectar and pollen brought into the beehives [Brodschneider
460 et al., 2010]. These results show how honey stores and even more so pollen stores,
461 declined at the end of the period the beehives were located in the sunflower fields as a
462 consequence of the end of the flowering period and isolation from other foraging
463 sources. The data suggest that the flowering period was short and that the bees had to
464 consume a large portion of the reserves stored over those days. The situation was
465 similar for all treatments, with no statistical significant differences between them (one-
466 way ANOVA $p<0.05$). However, the development of food stores was more uneven
467 when comparing the different replications, which suggests a potential effect due to
468 location, as shown by the multivariate analysis of the honey and pollen stores for other
469 variables such as weight as well (**Table 3**).

470 **4. CONCLUSIONS**

471 The strength of the colonies, measured as adult bees per hive, was a main factor for the
472 survival of the colonies in this study, which developed under a worst-case scenario. The
473 comparison of colonies of different strengths enabled us to confirm that the pattern in
474 the development of the colonies was the same, regardless of the treatment. The
475 development of the beehives was heavily affected by the environmental conditions
476 which, together with the initial strength of the beehives and the characteristics of the
477 plots, had a significant effect on the different variables studied. The concentration of
478 neonicotinoid residues in samples of bee bread and in adult bees was in the range of
479 0.10 – 2.89 $ng.g^{-1}$ and 0.05 – 0.12 $ng.g^{-1}$; 0.10 – 0.37 $ng.g^{-1}$ and 0.01 – 0.05 $ng.g^{-1}$, for

clothianidin and thiamethoxam, respectively. No significant differences have been found between control colonies and the colonies exposed to sunflower treated with, thiamethoxam or clothianidin.

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References:

Accorti, M., Persano, L., Piazza, M.G., Sabatini, A.G., 1986. Schede di caratterizzazione delle principali qualitá di mieli Italian. Apicoltura 2, 3-35.

AEMET (Agencia Española de Meteorología) (2011). Iberian climate atlas. Ed. Ministerio de Medio Ambiente y Medio Rural y Marino. Madrid (Spain), pp 80.

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. doi: 10.1002/ps.4583.

506 Boi, M., Serra, G., Colombo, R., Lodesani, M., Massi, S., Costa, C., 2015. A 10 year
507 survey of acaricide residues in beeswax analysed in Italy. Pest Manag. Sci. 72,
508 1366–1372.

509 Brodschneider, R., Crailsheim, K., 2010. Nutrition and health in honey bees. Apidologie
510 41, 278–294.

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

513 Carreck, N.L., Ratnieks, F.L.W., 2014. The dose makes the poison: have "field
514 realistic" rates of exposure of bees to neonicotinoid insecticides been
515 overestimated in laboratory studies? J. Apic. Res. 53, 607-614. doi
516 10.3896/IBRA.1.53.5.08

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

520 Chauzat, M.P., Faucon, J.P., 2007. Pesticide residues in beeswax samples collected
521 from honey bee colonies (*Apis mellifera L.*) in France. Pest. Manag. Sci. 63,
522 1100–1106.

523 Collison, C., 2016. Pollen Quality. Bee Culture. August 2016.
524 http://www.beeculture.com/pollen-quality/

525 Cutler, G. C., Scott-Dupree, C. D., Sultan, M., MacFarlane, A. D., Brewer, L., 2014. A
526 large-scale field study examining effects of exposure to clothianidin seed-
527 treated canola on honey bee colony health, development, and overwintering
528 success. Peer J2, 2:e652. doi: 10.7717/peerj.652

529 Dietemann, V., Nazzi, F., Martin, S.J., Anderson, D.L., Locke, B., Delaplane, K.S.,
530 Wauquiez, Q., Tannahill, C., Frey, E., Ziegelmann, B. (2013) Standard
531 methods for varroa research. J. Apic. Res. **52**(1), 1–54

532 Doull, K. M., 1976. The effects of different humidities on the hatching of the eggs of
533 honeybees. Apidologie 7, 61-66.

534 Erdtman, G (1960). The acetolysis method. SvenskBotaniskTidskrift 54: 561-564.

EFSA Guidance Document on the risk assessment of plant protection products on bees (*Apis mellifera*, *Bombus* spp. and solitary bees) 2013, EFSA Journal 11, 3295. doi: 10.2903/j.efsa.2013.3295

EPPO 170a, European and Mediterranean Plant Protection Organisation. Guidance "Efficacy evaluation of plant protection products. Side-effects on honeybees"

Eurostat (European Commission) 2017. Crop statistics (from 2000 onwards). Sunflower seed. http://appsso.eurostat.ec.europa.eu/nui/submitViewTableAction.do

Flores, J.M., Ruíz, J.A., Afonso, S.M., 2002. Assessment of the population of *Varroa destructor* based on its collection from boards at the bottom of hives of *Apis mellifera iberica*. Rev. Port. Ciênc. Vet. 97, 193-196.

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.

Hatjina, F., Costa, C., Büchler, R., Uzunov, A., Drazic, M., Filipi, J., Charistos, L., Ruottinen, L., Andonov, S., Meixner, M. D., Bienkowska, M., Dariusz, G., Panasiuk, B., Le Conte, Y., Wilde, J., Berg, S., Bouga, M., Dyrba, W., Kiprijanovska, H., Korpela, S., Kryger, P., Lodesani, M., Pechhacker, M., Petrov, P., Kezic, N., 2014. Population dynamics of European honey bee genotypes under different environmental conditions. J. Apic. Res. 53, 233–247.

Imdorf, A., Gerig, L., 2001. Course in determination of colony strength. Swiss Federal Dairy Research Institute, Liebefeld CH-3003 Bern Switzerland (after L Gerig, 1983. LehrgangzurErfassung der Volksstärke). SchweizBienen-Zeitung 106, 199-204.

Johnson, R.M., Ellis, M.D., Mullin, C.A., Frazier, M., 2010. Pesticides and honey bee toxicity USA. Apidologie 41,312-331. doi: org/10.1051/apido/2010018.ç

Khoury, D.S., Barron, A.B., Myerscough, M.R., 2013 .Modelling Food and Population Dynamics in Honey Bee Colonies. PLoS ONE 8: e59084. doi: 10.1371/ journal.pone.0059084

565 Khoury, D.S., Myerscough, M.R., Barron, A.B., 2011. A Quantitative Model of Honey
566 Bee Colony Population Dynamics. PloSone 6, e18491.

567 Lecocq, A., Kryger, P., Vejsnæs, F., Bruun Jensen, A., 2015.Weight Watching and the
568 Effect of Landscape on Honeybee Colony Productivity: Investigating the
569 Value of Colony Weight Monitoring for the Beekeeping Industry. PLoS ONE
570 10: e0132473. doi:10.1371/journal.pone.0132473

571 Loveaux, J., Maurizio, A., Vorwohl, G., 1978. Methods of melissopalynology. Bee
572 World 59, 139-157.

573 Lundin, O., Rundlöf, M., Smith, H.G., Fries, I., Bommarco, R., 2015. Neonicotinoid
574 Insecticides and Their Impacts on Bees: A Systematic Review of Research
575 Approaches and Identification of Knowledge Gaps. PLoS ONE 10: e0136928.
576 doi:10.1371/journal.pone.0136928.

577 Mateo, R., Bosch-Reig, F., 1998. Classification of Spanish unifloral honeys by
578 discriminant analysis of electrical conductivity, color, water content, sugars
579 and pH. J. Agric. Food Chem. 46, 393-400.

580 Meier, U., 2001. Growth stages of mono-and dicotyledonous plants – BBCH
581 Monograph. The BBCH codes are on:
582 http://www.reterurale.it/downloads/BBCH_engl_2001.pdf

583 Nicolson, S.W., Human, H., 2013.Chemical composition of the 'low quality' pollen of
584 sunflower (*Helianthus annus*, Asterarceae) Apidologie 44, 144–152.

585 Pérez, C., Burgaz, M.E., Peris, J.B., Duprè, M., 1994. Contribución al estudio de la miel
586 de girasol de Castilla-La Mancha. In: Trabajos de palinología básica y
587 aplicada (editado por I.A. Mateu). Pp. 79–86. Valencia: X Simposio de
588 Palinología (A.P.L.E.), Universidad de Valencia.

589 Pettis, J. S., Rice, N., Joselow, K., vanEngelsdorp, D., Chaimanee, V., 2016. Colony
590 failure linked to low sperm viability in honey bee (*Apis mellifera*) queens and
591 an exploration of potential causative factors. PLoS One, 11, e0147220.
592 doi:10.1371/journal.pone.0147220

593 Pilling, E., Campbell, P., Coulson, M., Ruddle, N., Tornier, I., 2013. A four-year field
594 program investigating long-term effects of repeated exposure of honey bee
595 colonies to flowering crops treated with thiamethoxam. PLoS One 8:e77193

596 Pisa, L.W., Amaral-Rogers, V., Belzunces, L.P., Bonmatin, J.M., Downs, C.A.,
597 Goulson, D., Kreutzweiser, D.P., Krupke, C., Liess, M., McField, M.,
598 Morrissey, C.A., Noome, D.A., Settele, J., Simon-Delso, N., Stark, J.D., Van
599 der Sluijs, J.P., Van Dyck, H., Wiemers, M., 2015. Effects of neonicotinoids
600 and fipronil on non-target invertebrates. Environ. Sci. Pollut. Res. Int. 22, 68–
601 102.

602 Potts, S.G., Biesmeijer, J.C., Kremen, C., Neumann, P., Schweiger, O., Kunin, W.E.,
603 2010. Global pollinator declines: trends, impacts and drivers. Trends Ecol.
604 Evol. 25, 345–353.

605 Regulation EU No 1107/2009 concerning the placing of plant protection products
606 on the market and repealing Council Directives 79/117/EEC and
607 91/414/EEC

608 Regulation EU No 485/2013 amending Implementing Regulation (EU) No 540/2011, as
609 regards the conditions of approval of the active substances clothianidin,
610 thiamethoxam and imidacloprid, and prohibiting the use and sale of seeds
611 treated with plant protection products containing those active substances. OJ L
612 139:12–26

613 Rolke, D., Fuchs, S., Grünewald, B., Gao, Z., Blenau, W., 2016. Large-scale monitoring
614 of effects of clothianidin-dressed oilseed rape seeds on pollinating insects in
615 Northern Germany: effects on honey bees (*Apis mellifera*). Ecotoxicology 25,
616 1648-1665.

617 Royal Decree 608/2006, of May 19, which establishes and regulates a national program
618 for the control of diseases of honey bees.

619

620 Sánchez-Bayo, F., 2014. The trouble with neonicotinoids. Chronic exposure to widely
621 used insecticides kills bees and many other invertebrates. Science 346, 806-
622 807.

623 Sanchez-Bayo, F., Goka, K., 2014. Pesticide Residues and Bees – A Risk Assessment.
624 PLoS ONE 9: e94482. doi:10.1371/journal.pone.0094482

625 SANCO/12571/2013. Guidance document on analytical quality control and validation
626 procedures for pesticide residues analysis in food and feed. Avalailable

at:http://ec.europa.eu/food/plant/plant_protection_products/guidance_documents/docs/qualcontrol_en.pdf.

Sandrock, C., Tanadini, M., Tanadini, L.G., Fauser-Misslin, A., Potts, S.G., Neumann P., 2014. Impact of Chronic Neonicotinoid Exposure on Honeybee Colony Performance and Queen Supersedure. PLoS ONE 9: e103592. doi:10.1371/journal.pone.0103592Sawyer, R., 1988. Honeydew. In: Honey Identification. Cardiff Academic Press, 30-32.

Schulz, D.J., Huang, Z.Y., Robinson, G.E., 1998. Effects of colony food shortage on behavioral development in honey bees. Behav. Ecol. Sociobiol. 42, 295–303.

Serra-Bonvehí, J., Orantes-Bermejo, J., 2010. Acaricides and their residues in Spanish commercial beeswax. Pest. Manag. Sci. 66:1230–1235.

Smith, K. M., Loh, E. H., Rostal, M. K., Zambrana-Torrelio, C. M., Mendiola, L., Daszak, P., 2013. Pathogens, Pests, and Economics: Drivers of Honey Bee Colony Declines and Losses. EcoHealth 10, 434–445. doi: 10.1007/s10393-013-0870-2

Schmuck, R., Lewis, G., 2016. Review of field and monitoring studies investigating the role of nitro-substituted neonicotinoid insecticides in the reported losses of honey bee colonies (*Apis mellifera*). Ecotoxicology 25, 1617–1629.

Terrab, A., Díez, M.J., Heredia, F.J., 2003. Palynological, physico-chemical and colour characterization of Moroccan honeys: III. Other unifloral honey types. Int. J. Food Sci. Tech. 38, 395-402.

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.

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,

658 L., Sárospataki, M., Saure, C., Edwards, M., Genersch, E., Knäbe, S., Pywell,
659 R.F., 2017. Country-specific effects of neonicotinoid pesticides on honey bees
660 and wild bees. Science 356, 1393-1395.

Table 1a. Evolution of the adult bee population (mean ± s.d.) corresponding to the colonies surviving until the end of the study (n = number of colonies), able to overcome winter and recover, observed for the different treatments in each evaluation -A1 to A6-.

Number of adult bees in each evaluation							
Treatments	n	**A 1**	**A 2**	**A 3**	**A 4**	**A 5**	**A 6**
T1	20	16343.5±4231.9	15781.7±4182.7	13354.6±3401.6	7993.4±2056.8	9169.7±4138.5	18710.8±5467.2
T2	18	15707.6±4605.8	15117.1±3323.4	12878.5±3560.6	8585.1±2345.8	10000.9±4235.6	18632.2±7337.7
T3	17	15954.4±3560.8	14742.4±4636.5	12870.5±4348.9	7592.4±2643.5	8907.4±4513.1	18240.1±7663.1
Percentage change in the number of bees with respect to the previous evaluation							
Treatments	n		**% A1 to A2**	**% A2 to A3**	**% A3 to A4**	**% A4 to A5**	**% A5 to A6**
T1	20		-3.44	-15.38	-40.14	+14.72	+104.05
T2	18		-3.76	-14.81	-33.34	+16.49	+86.30
T3	17		-7.60	-12.70	-41.01	+17.32	+104.78
Percentage change in the number of bees with respect to the first evaluation							
Treatments	n			**% A1 to A3**	**% A1 to A4**	**% A1 to A5**	**% A1 to A6**
T1	20			-18.29	-51.09	-43.89	+14.48
T2	18			-18.01	-45.34	-36.33	+18.62
T3	17			-19.33	-52.41	-44.17	+14.33

T1: control; T2: thiamethoxam; T3: clothianidin

Note: In the second and third batches, the % change in the number of adult bees in each evaluation is shown with respect to the previous evaluation and with respect to the first evaluation, A1 (the negative sign indicates a decline in the number of bees, and the positive sign an increase in the number of bees).

Table 1b. Number of dead colonies in each treatment, strength (number of adult bees) when placed in the flowering sunflower fields.

Quartile	**1st < 13197**		**2nd 13197 to 15498**		**3rd 15498 to 18156**		**4th > 18157**	
Nº of bees (mean ± s.d.)	9944.50 ± 2121.27		14560.00 ± 585.71		16793.78 ± 817.02		20549.33 ± 1815.74	
Treatments	**nº of colonies**	**dead colonies**	**nº of colonies**	**dead colonies**	**nº of colonies**	**dead colonies**	**nº of colonies**	**dead colonies**
T1	5	1	3	1	10	2	6	0
T2	6	3	9	3	4	0	5	0
T3	7	2	6	2	4	1	7	2
Total (nº of colonies)	18	6	18	6	18	3	18	2

T1: control; T2: thiamethoxam; T3: clothianidin

The colonies are grouped in quartiles according to the number of adult bees recorded in evaluation 1 (A1) before relocating the bees to the flowering fields. The range of each quartile and the average number (mean ± s.d.) of bees can be seen at the top.

Table 2. Correlation analysis, Pearson correlation test, for the various variables observed in the bee colonies.

		hive weight	stored honey (cm^2)	stored pollen (cm^2)	drone brood	worker brood
Nºof adult bees	Pearson correlation test	.686**	.510**	.391**	.383**	.645**
	Sig. (bilateral)	.000	.000	.000	.000	.000
	N	475	475	475	475	475
worker brood	Pearson correlation test	.568**	.322**	.458**	.232**	
	Sig. (bilateral)	.000	.000	.000	.000	
	N	475	475	475	475	
drone brood	Pearson correlation test	.438**	.383**	.087		
	Sig. (bilateral)	.000	.000	.059		
	N	475	475	475		
stored pollen (cm^2)	Pearson correlation test	.432**	.349**			
	Sig. (bilateral)	.000	.000			
	N	475	475			
stored honey (cm^2)	Pearson correlation test	.896**				
	Sig. (bilateral)	.000				
	N	475				

Table 3. Univariate analysis *of* variance*:* effect of location, number of adult bees in the colonies when were placed in the flowering sunflower fields (grouped into quartiles), and treatment, as well as the interaction of the former two with the treatment, on the different variables studied in the beehives. Confidence interval, 95% (p values) in each evaluation (A1 to A6).

		Replication	Treatment	Replication x Treatment	Quartile	Treatment	Quartile x Treatment
hive weight	**A1**	0.146	0.447	0.874	0.000	0.592	0.265
	A2	0.002	0.688	0.937	0.000	0.569	0.649
	A3	0.003	0.094	0.928	0.000	0.074	0.635
	A4	0.000	0.174	0.861	0.000	0.229	0.849
	A5	0.000	0.204	0.665	0.000	0.109	0.703
	A6	0.021	0.620	0.549	0.000	0.291	0.493
nº adult bees	**A1**	0.241	0.543	0.987	0.000	0.592	0.265
	A2	0.201	0.201	0.947	0.000	0.569	0.649
	A3	0.094	0.495	0.876	0.000	0.074	0.635
	A4	0.387	0.176	0.693	0.000	0.229	0.849
	A5	0.019	0.494	0.759	0.001	0.109	0.703
	A6	0.066	0.904	0.333	0.051	0.291	0.493
worker brood	**A1**	0.057	0.257	0.588	0.002	0.449	0.691
	A2	0.791	0.267	0.462	0.008	0.460	0.570
	A3	0.198	0.603	0.718	0.006	0.815	0.631

	A4	0.153	0.138	0.157	0.042	0.186	0.001
	A5	0.370	0.298	0.267	0.005	0.157	0.370
	A6	0.023	0.835	0.568	0.003	0.983	0.699
stored honey	**A1**	0.155	0.331	0.503	0.000	0.569	0.253
	A2	0.000	0.656	0.779	0.000	0.599	0.683
	A3	0.000	0.125	0.869	0.001	0.120	0.968
	A4	0.000	0.485	0.741	0.000	0.554	0.820
	A5	0.001	0.203	0.703	0.000	0.165	0.821
	A6	0.091	0.479	0.611	0.000	0.207	0.140
stored pollen	**A1**	0.453	0.118	0.098	0.335	0.355	0.159
	A2	0.010	0.063	0.002	0.002	0.029	0.032
	A3	0.000	0.112	0.095	0.069	0.198	0.62
	A4	0.155	0.355	0.577	0.95	0.339	0.853
	A5	0.001	0.295	0.048	0.263	0.316	0.049
	A6	0.06	0.704	0.072	0.024	0.354	0.108

Highlights

Madrid, December, 2017

Dear Editor,

The highlights of the work entitled: **"Viability of honeybee colonies exposed to sunflowers grown from seeds treated with the neonicotinoids thiamethoxam and clothianidin"** are:

This work is presented as a field study for high tier evaluations

It is evaluated the impact of neonicotinoids on colonies of honeybees

It was conducted on sunflowers, traditionally used for beekeeping in Spain

The data have been generated in the temperate/warm climate of southern Europe.

Thank you for your consideration of this paper.

Looking forward to hearing from you,

Sincerely,

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