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Thiamethoxam: Long-term effects following honey bee colony-level exposure and implications for risk assessment

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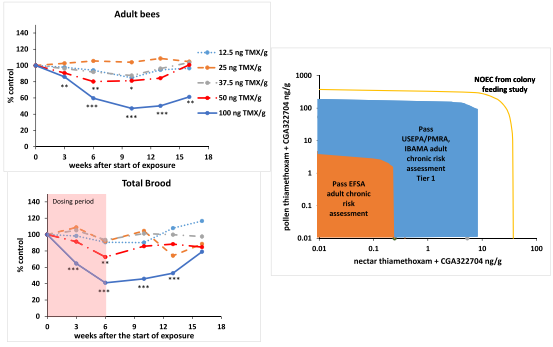
HIGHLIGHTS

- Study aimed to determine long-term impact of dosing (6 weeks) thiamethoxam (TMX).
- Dosing 100 ng TMX/g feed resulted in significant colony-level adverse effects.
- Significant effects occurred after dosing with 50 ng TMX/g feed, but colonies recovered within 2–3 brood cycles.
- At ≤37.5 ng TMX/g feed there were no colony-level effects before or after winter.
- EU risk assessment requires field studies at residues >30 fold lower than NOEC.

GRAPHICAL ABSTRACT



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ABSTRACT

Neonicotinoid insecticides have been used in a wide range of crops through seed treatment, soil and foliar applications and a large database exists on both their lethal and sub-lethal effects on honey bees under controlled laboratory conditions. However, colony-level studies on the effects of neonicotinoids in field studies are limited, primarily due to their complexity and the resources required. This paper reports the combined results of two large-scale colony-feeding studies, each with 6 weeks of continuous dosing of 12 colonies per treatment (24 control) to 12.5, 25, 37.5, 50 or 100 ng thiamethoxam/g sucrose solution. Exposure continued beyond dosing with residues present in stored nectar and bee-bread. The studies were conducted in an area with limited alternative forage and colonies were required to forage for pollen and additional nectar The studies provide colony-level endpoints: significant effects (reductions in bees, brood) were observed after exposure to the two highest dose rates, colony loss occurred at the highest dose rate, but colonies were able to recover (2–3 brood cycles after the end of dosing) after dosing with 50 ng thiamethoxam/g sucrose. No significant colony-level effects were observed at lower dose rates. The data reported here support the conclusions of previous colony-level crop-based field studies with thiamethoxam, in which residues in pollen and nectar were an order of magnitude below the colony-level NOEC of 37.5 ng thiamethoxam/g sucrose. The feeding study data are also compared to the outcomes of regulatory Tier 1 risk assessments conducted using guidance provided by the USA, Canada, Brazil and the EU regulatory authorities. We propose an adaptation of the European chronic adult bee risk assessment that takes into account the full dataset

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generated in laboratory studies while still providing an order of magnitude of safety compared with the colony feeding study NOEC.

1. Introduction

Neonicotinoid insecticides have been used globally on a wide range of crops through seed treatment as well as soil and foliar applications (Simon-Delso et al., 2015). Due to their widespread use and systemic properties, resulting in their detection in pollen and nectar of bee attractive crops, this class of insecticides has been extensively studied under laboratory conditions to assess a wide range of sub-lethal effects on bees (Pisa et al., 2014; Stephenson and Solomon, 2017). The aim of regulatory risk assessments is to determine if protection goals, which for honey bees can include pollination services, hive product production and biodiversity, are compromised (USEPA, 2014). For honeybees, the relevant endpoints are colony-level assessments (development and survival) or surrogate laboratory endpoints. Available field studies on the effects of neonicotinoids at the colony-level are limited, primarily due to the resources required and their complexity, e.g. plot separation, absence of alternative forage and scale of replication (Woodcock et al., 2017). Although such field studies reflect real-use conditions and can be used in a weight of evidence approach for risk characterization, expert judgement is required for their interpretation; as the field not the hive is the replicate field studies usually have low statistical power and thus they have been open to criticism (Campbell et al., 2017; Schick et al., 2017). An alternative approach which addresses many of the limitations of field studies is to use colonies directly fed with treated feed in the absence of alternative forage (Dively et al., 2015; Faucon et al., 2005; Odemer and Rosenkranz, 2018; Seide et al., 2018; Thompson et al., 2014). Such colony-feeding studies enable exposure to be controlled with a concentration-response relationship defined at the colony-level and improved statistical power to detect effects on colony development; the colony being considered the replicate and therefore, within practical considerations, more colonies can be used to increase power. This study design provides a means of determining exposure thresholds below which the likelihood of adverse effects (irreversible effects on numbers of adult bees or brood or on colony survival) on colonies is low (USEPA, 2016). A previous colony feeding study conducted in 2014 as part a regulatory data package (Overmyer et al., 2018), reported the results of a honey bee colony feeding study which demonstrated no adverse effects after dosing with 50 ng thiamethoxam/mL sucrose but overwintering success was poor across all treatments and controls due to delayed autumn feeding of the colonies. This paper reports the results of a second thiamethoxam colony feeding study (conducted in 2016 as part of a regulatory data package) which mirrored the design of the first but included normal beekeeping practice of supplemental feeding in autumn as required to permit colonies to over-winter successfully. Colonies were dosed for 6 weeks and then exposed to residues in food stores within the hive through the over-wintering period (which started 10–11 weeks after the end of dosing) to the following spring. As the studies were comparable in location, climate conditions and source of bees, the pre-winter data from both studies were analysed together in order to maximise power to detect any effects of thiamethoxam exposure on honey bee colonies. The over-wintering data from the second study were also subjected to analysis.

There have been concerns in Europe raised over the potential risks to bees foraging both on seed-treated crops and on crops planted in fields in which thiamethoxam has been used in the previous season, e.g. oilseed rape (*Brassica napus*) following seed-treated sugar-beet (EFSA, 2018). The colony feeding study data provide a robust colony-level endpoint which can then be compared with pollen and nectar residue data from both seed-treated crops and follow-on crops; these data can also be used to further characterize the outcomes of the United States Environmental Protection Agency/Health Canada Pest Management Regulatory Agency (USEPA/PMRA) and European Food Safety Authority (EFSA) first tier (Tier 1) risk assessments for thiamethoxam seed-treated crops.

2. Methods

2.1. Colony feeding study

The study was based on the same design as that conducted in 2014 (Overmyer et al., 2018). This allowed for increased power in statistical analysis by combining colony strength data from the two studies for the 16–17 weeks of exposure until the start of the over-wintering period (27–29 October 2014, 24 Oct–2 November 2016). The apiaries for both studies were situated in Orange, Caswell or Alamance Counties, in central North Carolina, USA, an area primarily composed of forested and pasture land with some small tracts of crops such as tobacco, corn, and soybeans (flowers are removed from tobacco in normal agronomic practice, corn produces only pollen and soybeans are not considered highly attractive to honeybees); therefore, the potential for exposure of honey bees to pesticide via treated crops was relatively low. During the 6-week dosing phase, temperature (daily minimum/maximum), relative air humidity (daily minimum/maximum), and rainfall were recorded from weather stations at two sites (0.4 to 14.8 km from the apiaries).

Honey bees (*Apis mellifera ligustica*, the sub-species of honey bee used by most commercial beekeepers in the south-eastern United States) were purchased as packages from a commercial bee supplier (J J's Honey, Georgia, USA). The bees were installed in new hive boxes (10-frame Langstroth boxes) at the beginning of April 2016 with a new queen (supplied by the same source but they were not sister queens, due to the number of colonies required) introduced into each colony. Over 100 colonies were assessed to determine the numbers of adult bees and numbers of cells containing brood, pollen/bee-bread and nectar/honey in early June (4 weeks before the start of exposure (4 WBE)) (Imdorf et al., 1987; Imdorf and Gerig, 1999) and of these, 96 colonies were selected for the study based on general health. Colonies had all stages of brood, a queen, and some food stores but no visible symptoms of *Varroa mites* (*Varroa destructor*), *Nosema* or other bee diseases. Between 24 June and 1 July 2016 (1 to 2 weeks before the start of the exposure (dosing) period; (1–2 WBE)) the colonies were visually assessed again; each colony consisted of one to two brood boxes (10 frames in each box), 5000–35,000 adult bees, 5 to 17 brood combs with all brood stages and 8–24 combs containing nectar, honey and bee-bread (stored, processed pollen with honey added).

After the first pre-exposure assessment at 4 WBE the colonies were moved from the holding yards to the study apiary site locations. Each of the 96 colonies was allocated to one of the 12 study apiary sites (A–L) (all apiaries were at least 1 km apart) by stratifying according to colony strength (apiary A had the strongest colonies (1400 $\pm$ 300 adult bees); apiary L had the weakest colonies (8300 $\pm$ 40 adult bees)). At every apiary site each hive within an apiary was raised on a pallet above ground level and spatially separated from other hives by at least 9 m. Hives were arranged in a semi-circular pattern, facing east to west, with 38 m spacing between the two hives at the ends of the semi-circle to minimize drifting and deter robbing. The 8 hives were then randomly allocated to treatment: 7 hives for biological assessments (2 untreated

and one for each of the 5 thiamethoxam treatment levels) and one monitoring hive which served as a method of sampling of both what the bees were foraging on within the landscape and of potential pesticide exposure from other sources.

With the exception of the monitoring hive, all hives were provided untreated sucrose solution (50% w/w in tap water) or sucrose solution (50% w/w in tap water) containing 12.5, 25, 37.5, 50 or 100 ng thiamethoxam/g (technical 98.9% purity supplied by Syngenta, LLC) by preparing a stock solution of 0.038 g of thiamethoxam dissolved in approximately 20 mL of acetone, diluting with tap water to 1000 mL (38 mg/1000 g = 38 ppm w/w) and then further diluting with sucrose solution to the appropriate test concentration and volume.

The exposure period started during the first week of July 2016 (6 July 2016) and dosing ended in mid-August 2016. Untreated or treated sucrose (2 L) was supplied twice per week for 6 weeks in an internal feeding source (division board frame feeder) placed at the edge of the upper brood box within the hive. At each renewal, any solution remaining from the previous feeding was removed and measured and freshly prepared sugar solution was provided. Dosing solution concentrations were determined from samples taken at the time of dosing. Dosing solution stability was also determined under hive conditions 1 week and 5 weeks after initiation of the exposure period by placing samples of each dosing solution, sealed in 15-mL Falcon tubes, in monitoring hives at each apiary site (they were not available to the colony). These stability samples were removed at the next feeding event and stored frozen.

Colony condition assessments (CCAs) were conducted to observe the overall colony performance. Due to the size of the study, each CCA period took several days to complete and during the assessments, colonies were also checked for visible signs of disease or pests, such as *Nosema*, European foulbrood (*Melissococcus plutonius*), American foulbrood (*Paenibacillus larvae*), *Varroa* mites or small hive beetle (*Aethina tumida*). Assessments were conducted from before the start of exposure on 24 Jun–01 Jul 2016 (1–2 WBE), 19–30 Jul 2016 (2–3 weeks after the start of exposure (WAE)), 13–18 Aug 2016 (5–6 WAE), 12–16 Sep 2016 (10 WAE), 03–06 Oct 2016 (13 WAE), 24 Oct–02 Nov 2016 (16–17 WAE) and twice after overwintering on 06–09 Mar 2017 (35 WAE) and 03–07 Apr 2017 (39 WAE). To prevent colony loss due to starvation and in accordance with local beekeeping practices, all colonies in 2016 were fed with 3 L of sucrose solution (2:1 sucrose:water) on 4 occasions from mid-September to mid-October 2016 in preparation for overwintering. This coincides with typical colony maintenance feeding with sucrose solution which starts in August in the Piedmont area of North Carolina where this study was conducted.

Assessments of *Varroa* mite abundance and *Nosema* spore levels were assessed in the colonies once before start of exposure (1–2 WBE), at the end of the dosing period (5–6 WAE), twice in fall before overwintering (10 WAE, 16–17 WAE) and once after overwintering (34 WAE). For *Varroa* assessments, samples of bees were collected from each colony, counted and washed in alcohol to remove and count mites. The number of mites per 100 bees was calculated. Two prophylactic *Varroa* treatments with Apiguard® (active ingredient: 25% thymol) were performed at an appropriate timing at the end of summer, 23–25 Aug 2016 (1st treatment, 50 ml Apiguard®) and 02 Sep 2016 (2nd treatment, 50 ml Apiguard®), and a third treatment was a Mite-Away-Quick Strip (MAQS®) application (active ingredient: formic acid) made on 10 and 11 Oct 2016 after the assessments at 13 WAE. For the *Nosema* spore assessments, the abdomens of 30 bees were removed and ground with distilled water in a mortar; a drop of diluted suspension was placed on a hemocytometer and spores counted using a microscope (×400 magnification).

During the CCAs, observations were recorded for each side of each frame of the hive with the percentage of area represented by adult bees, nectar/honey, bee-bread, capped brood, larvae, and eggs (Imdorf et al., 1987; Imdorf and Gerig, 1999) recorded by trained experts. The number of adult bees within the hive was estimated by recording the percentage area of the frame covered and the total bee population present within the hive based on a density of 130 bees per 100 cm^2 (based on counting bees in photographs). In the 2014 study (Overmyer et al., 2018) for all assessments the numbers of bees in the hive at the time of the observation were both visually assessed and assessed using image analysis. This demonstrated that although visual assessments were on average 20% lower than the image analysis there was a consistent correlation between visual and digital assessments ($r^2 = 0.91$) (see Supplementary Data). As the visual assessments in 2014 were less disruptive to the colony, only visual observations were used in the 2016 study. The total number of cells per frame is 3440 (based on 4 cells per cm^2 and 860 cm^2 per comb side); therefore, to estimate the total number of cells of brood or food stores, the percentage coverage was multiplied by 3440 for one side of a frame. In 2014 the visual assessments of comb cell numbers were validated by comparison with digital assessments for 5 colonies (see Supplementary Data). This more limited validation of cell numbers was due to the required removal of all bees from every side of frames for digital cell assessments. Removal of adult bees from frames is extremely disruptive and time consuming resulting in potential impacts on the colony due to the time it requires the colony to be opened, potentially resulting in robbing, and damaging adult bees during removal by shaking or brushing.

Due to the number of colonies needing to be assessed, the apiaries were split between two experts for the CCAs. Each expert only assessed hives in their six designated apiaries to reduce any potential added variability as a result of the different experts conducting the CCAs. The hives were assessed in the order they were placed in the semi-circle, i.e. not in treatment order. During the colony condition assessments, and all other hive inspections in between (e.g. for feeding, *Varroa* treatment, hive expansion or removal of boxes), any indications for potential swarming were checked and noted. In the pre-wintering assessments any queen cells were removed and a hive was expanded to reduce swarm pressure if considered appropriate by the beekeeper. Unfortunately in the post-wintering assessments swarming had already occurred at the first colony check. The weight of each hive was also recorded manually twice per month, starting in June 2016 until the last colony assessment in April 2017.

Samples of bee-bread and nectar were collected from all hives before the exposure phase, 6 weeks after the start of exposure, i.e. at the end of dosing, and after overwintering (35 WAE) for analysis of thiamethoxam and its major metabolite CGA322704 (clothianidin). Samples of bee-bread (at least 500 mg total) were collected from at least 5 different areas of frames per colony using pollen corers and pooled per colony. Nectar samples (at least 500 mg total) were collected from at least 5 different frames (if available) per colony with a single use plastic spoon or other suitable clean tool.

Samples of pollen and nectar were also collected from the monitoring hive at each apiary pre-exposure and 1, 3, 5, 6, 10, 13 and 16 weeks after start of exposure of the treated hives. Pollen traps were placed at the entrances of all monitoring hives and remained activated (pollen grids closed) for approximately 24–48 h for each sampling point. The duration of pollen trap activation was adapted to pollen availability. Pollen samples were split into 2 sub-samples: one for pollen identification and one for residue analysis (pesticide screening; analysed for 174 active ingredients using multi-residue methods). As external pollen availability varied, pollen amounts collected from each hive pollen trap were variable and samples were not available from every site at each collection event.

All samples collected for residue analysis and pollen identification were placed in freezer storage as soon as possible after collection. All samples for residue analysis were shipped to EPL Analytical Laboratory (Niantic, IL, USA) where analysis was conducted for thiamethoxam and CGA322704; the samples for multi-residue pesticide screening were shipped from EPL to the USDA Laboratory (Gastonia, NC, USA). Samples

for pollen identification were shipped to the Nds. Landesamt für Verbraucherschutz und Lebensmittelsicherheit (LAVES), Institut für Bienenkunde, Celle, Germany.

2.2. Analytical methods

Pollen analysis for screening of a wide range of pesticides (and thymol) was undertaken by the USDA Laboratory (Gastonia, NC, USA) using multi-residue methodology described in Mullin et al. (2010).

Sucrose used for treatments and stability assessments, bee-bread and nectar were analysed for thiamethoxam and metabolite CGA322704 according to the methodology described in Overmyer et al. (2018). Methods were verified prior to analysis of samples (all recoveries were within 80–120% and RSD <20%). Spiked sample recoveries performed in parallel with sample analysis were 71.3–112% and 72.5–115% for thiamethoxam and CGA322704 respectively in bee-bread and 93.1–119% and 86.0–113% for thiamethoxam and CGA322704 respectively in nectar and sucrose. The Limits of Quantitation (LOQs) for both analytes were 0.5 ng/g for nectar and 1 ng/g for bee-bread; the Limits of Detection (LODs) were 50% of the respective LOQ.

2.2.1. Pollen source identification

For details of pollen identification methods see Overmyer et al. (2018). In outline, an aliquot of homogenized water-pollen solution was transferred onto a microscope slide, distilled water added, homogenized and spread. After drying, embedding took place with a drop of the mounting medium (Kaiser's Glycerine jelly) added under a cover slip. A microscope with magnifications of 400× and 1000× was used to identify the pollen grains to plant species. Pollen grains identified to species in each sample were counted in a minimum of five areas of the slide containing at least 100 pollen grains to calculate their proportion in the total pollen sample.

2.2.2. Data evaluation

The potential effects of thiamethoxam on honey bee colony health were evaluated by comparing the data from test item treatment colonies to the control colonies. For pre-wintering assessments, the 2016 data and 2014 data (Overmyer et al., 2018) were analysed together. While CCAs were conducted over multiple days, for summary statistics, the first day was used to characterize any given assessment period. Statistical analysis was conducted using SAS/STAT® software, Version 9.4 of the SAS System for Windows.

Any colony that did not show the presence of a queen and had no open brood or eggs, or was devoid of worker (female) bees was considered "dead". If a hive was considered "dead" at the time of assessment, it was no longer used in the analysis of endpoints (e.g., adult bee numbers, hive weight, etc.).

Initially, an analysis of covariance (ancova) model was fitted to the data for each parameter/assessment time with terms for year, apiary-within-year, treatment and the treatment-by-year interaction, and with the corresponding pre-treatment assessment included as a continuous covariate. For the over-wintering assessments where data were only collected in the 2016 study, the initial model for each parameter had terms for apiary and treatment, with the corresponding pre-treatment assessment included as a continuous covariate. The residuals arising from the model were plotted and their adherence to the assumptions underlying anova was checked. For the vast majority of the count parameter/assessment time combinations, there was clear evidence of a relationship between the fitted values and the variance of the residuals; this is not unusual for count data and pointed either to the need for a data transformation or for the application of a generalised linear model with non-normal error distribution. Usually the choice of transformation or error distribution should be heavily dependent on the nature of the biological process. However, because the data here are pseudo-counts rather than counts (they are converted from area covered to number and are therefore not a continuous distribution), the true form of the underlying distribution is uncertain and so there is no clear expectation as to the most appropriate transformation or error distribution. Having investigated various possibilities, a simple square-root transformation (applied to both the response data and the pre-treatment data) was found to be suitable for all the count parameter/assessment time combinations. This transformation was therefore used in all cases. The hive weight data were analysed untransformed.

As part of the design process, colonies were allocated to apiaries in a systematic way based on initial colony size. Consequently, some degree of confounding between the effect of apiary and the effect of the covariate (colony size) might be expected and this was indeed found to be the case. This raises the question of whether it makes sense to include both apiary and the colony size covariate in the model. Investigations revealed that including both terms was clearly beneficial in a number of cases as the residual variance and SEDs were lower compared to models including only one of the terms. Because retaining both terms was never detrimental, for consistency all parameter/assessment times were analysed with both terms included in the model.

The interaction between treatment and year was statistically significant at the 5% level for seven out of the 35 count parameter/assessment combinations with two years of data. For these parameter/assessment combinations by year analysis was conducted in addition to the across year analysis. The interaction between treatment and year was not statistically significant at the 5% level for any of the hive weight assessments.

The final analysis for each of the count parameter/assessment time combinations was therefore an ancova with the response as the pseudo-counts transformed with the square-root transformation. For the assessments with two years of data the model had terms for year, apiary-within-year, treatment and the treatment-by-year interaction, and the corresponding pre-treatment assessment square-rooted was included as a continuous covariate. For the over-wintering assessments where data were only collected in the 2016 study, the model had terms for apiary and treatment, and the corresponding pre-treatment assessment square-rooted was included as a continuous covariate.

The final analysis for each assessment time for the hive weight data was also an ancova but without any transformation of the response or the covariate. For the assessments with two years of data the model had terms for year, apiary-within-year, treatment and the treatment-by-year interaction, and the pre-treatment hive weight was included as a continuous covariate. For the over-wintering assessments where data were only collected in the 2016 study, the model had terms for apiary and treatment, and the pre-treatment hive weight was included as a continuous covariate.

For all analyses, within the model framework, the treatments were compared to the control using a one-sided Dunnett's test to assess for reductions compared to the control. As mentioned above, for the seven parameter/assessment combinations where the interaction between treatment and year was statistically significant at the 5% level, by year analyses were also considered. However in each case, the nature of the interaction was such that it did not seriously undermine the results of the across year analysis and so only these analyses are presented.

2.2.3. Residues in pollen and nectar of follow-on crops

Field sites where thiamethoxam treated sugar beet (0.6 mg thiamethoxam/seed) had been planted by farmers in spring 2016 were located in Groβ-Umstadt (medium clayey silt soil) and in Bergen (sandy loam soil) in Germany (Cruiser Force™ at 75 g ai/ha and 54 g ai/ha) respectively and in Remisbreite (clay-loam) in Austria (Cruiser 600 FS™ at 60 g ai/ha). In spring 2017, untreated follow-on crops representing a range of bee-attractive flowering crops were planted (spring oilseed rape, maize and *Phacelia*) to provide 3 subplots of each crop, each covered within a tunnel (108 m^2) to avoid cross-contamination. Based on recent published EFSA opinions stating

potatoes as a bee-attractive crop (EFSA, 2015; EFSA, 2016) these were also planted. Nectar was not collected as potatoes produce no nectar; pollen cannot be readily collected therefore anthers were sampled as a surrogate. Prior to planting of untreated follow-on crops, soil samples were collected for residue analysis; during flowering, soil, pollen (from all crops) and nectar (from *Phacelia* and oilseed rape) samples were also collected for residue analysis; pollen and nectar were collected three times: at the start, middle and end of flowering (for details see Supplementary Data Table S7).

2.2.3.1. Sample collection. For maize and potato, pollen and anthers, respectively, at least 200 mg were collected by hand from at least 12 plants, using a suitably distributive pattern across the plot. For maize pollen was collected by shaking the tassels over a sieve into a plastic bag. Potato anthers were collected by removing the anthers from the blossom with tweezers. For the collection of oilseed rape and phacelia pollen, a honey bee colony was placed in each tunnel at the start of flowering. The hives were closed at the beginning of each sampling day and bees were caught in front of the hives or directly from flowers across the entire tunnel. Bees were then placed in a box containing dry ice. Outside the tunnels the pollen sacs (at least 200 mg) were collected from the frozen/dead bees and placed into a vessel.

Honey bees were also used to collect nectar from oilseed rape and phacelia. The hives were closed at beginning of the sampling day and bees were caught in front of the hives or directly from flowers across the entire tunnel and placed in a box containing dry ice. A minimum of 20 bees were collected per tunnel. Outside the tunnels the honey-stomach was collected from the frozen/dead bees by dissecting the bees between thorax and abdomen. The honey-stomach contents were collected (at least 200 mg) and transferred to a vessel.

Soil was collected by hand, using a metal barrelled corer of 5 cm diameter to a depth of 25 cm across the entire plot, following a W-pattern.

All samples were placed in cooled conditions (gel packs) until transferred to the freezer (max −18 °C).

2.2.3.2. Analytical methods. Sub samples of soil (20 g) were extracted twice by shaking with 80/20 (v/v) acetonitrile/10 mM ammonium acetate at room temperature for 30 min each. The extracts were combined upon centrifugation and the volume reduced to approximately 20 mL by rotovap concentration. The concentrated extract was quantitatively transferred to a 50 mL centrifuge tube, volume adjusted to 50 mL with HPLC grade water (final composition contains approximately 10% methanol; v/v) and mixed well. An aliquot (1.5 mL) of the final soil extract was filtered through a PTFE syringe membrane filter (0.45 μm) to remove fine suspension particles prior to final dilution for subsequent residue determination. Subsequently, an aliquot of the final extract was diluted with 50 mM ammonium acetate/MeOH (90/10; v/v). For low level residue determination of CGA322704, sample extracts were taken through a solid phase extraction (SPE) procedure using Oasis MCX cartridges. Final residue determination was by high performance liquid chromatography with triple quadrupole mass spectrometric detection (LC-MS/MS) using electrospray ionisation techniques.

Matrix-matched standards were used for the quantification of thiamethoxam and CGA322704 in undiluted and diluted extracts of soil. Consequently a surrogate control matrix was used to prepare matrix matched standards and procedural recoveries. As sugar beet is typically grown in sandy loam soil, a sandy loam soil was used as a surrogate control (i.e. untreated) matrix.

The surrogate control matrix used to prepare the matrix matched standards and procedural recoveries contained no residue >LOD (limit of detection; 30% LOQ). LOQ 1.0 ng thiamethoxam/g soil, 0.1 ng CGA322704/g soil. Mean recovery in soil samples fortified at LOQ, 10 times LOQ and 25 times LOQ was 97% (RSD 9.7%) for thiamethoxam and 83% (RSD 14.2%) for CGA322704.

Pollen and nectar (100 mg) were extracted by vigorous shaking with methanol/0.2% formic acid in ultra-pure water (50/50, v/v). Aliquots equivalent to 50 mg were diluted with ultra-pure water. Sample clean-up was performed by solid-phase extraction (SPE) using Oasis HLB cartridges. Final determination was by high performance liquid chromatography with triple quadrupole mass spectrometric detection (LC-MS/MS).

Matrix-matched standards were used for the quantification of thiamethoxam and CGA322704 in undiluted extracts of pollen and nectar. Dilution of the extracts was not necessary.

A surrogate control matrix was used to prepare matrix matched standards and procedural recoveries. Maize pollen sourced from plots used for residue studies was used as a surrogate pollen control (i.e. untreated) matrix. A 36% sugar solution in ultra-pure water was prepared in the analytical laboratory for use as a surrogate nectar control (i.e. untreated) matrix.

The surrogate control matrix used to prepare the matrix matched standards and procedural recoveries contained no residue >LOD (limit of detection; 30% LOQ). LOQ: 0.5 ng thiamethoxam/g nectar; 1.0 ng thiamethoxam/g pollen; 1.0 ng CGA322704/g nectar; 1.0 ng CGA322704/g pollen. Mean recovery in pollen samples fortified at LOQ and 10 times LOQ was 99% (RSD 4.0%) for thiamethoxam and 88% (RSD 15.6%) for CGA322704. Mean recovery in nectar samples fortified at LOQ and 10 times LOQ was 100% (RSD 4.0%) for thiamethoxam and 89% (RSD 4.8%) for CGA322704.

3. Results

During the 6-week dosing period, environmental conditions were within expected ranges for the locality, and similar to during the 2014 study (Overmyer et al., 2018), with temperatures ranging from 19 °C to 36 °C and a total of 107 mm of rainfall accumulated (58 mm during July and 49 mm in the first 2 weeks of August). The minimum temperatures during the overwintering period were −5 °C in November, −9 °C in December, −17 °C in January, −6 °C in February and −6 °C in March.

The results for the 2014 study were reported in detail in Overmyer et al. (2018). Detailed information on colony development and environmental conditions in the 2016 study are provided in the Supplementary Data (Table S2) and summarised below. The 2014 and 2016 datasets up until the start of overwintering (16–17 weeks after the start of exposure) in each study were combined for analysis. The results of overwintering in the 2016 study are reported separately from the combined 2014 and 2016 datasets as insufficient and late feeding in 2014 resulted in high levels of colony loss in all treatments and control (Overmyer et al., 2018).

3.1. Colony-level exposure

The concentration of the test item measured in the sugar solution remained stable (within 20% nominal) at the two assessment points during the study in 2016. The mean consumption of sucrose by colonies during the 6-week dosing period was 95–100% of the total 24 L provided per hive. The total amount of sucrose supplied to the colonies in 2016 was higher than in the 2014 study (Overmyer et al., 2018). However, based on the mean size of the colonies (estimated numbers of adult bees) at the start of the dosing period the amount supplied per bee in each study was comparable (1.4–1.6 mL sucrose/adult bee).

Before exposure thiamethoxam and primary metabolite CGA322704 residues in all control and treated colonies were below the LOQ (0.5 ng/g). At the end of the dosing period no residues of thiamethoxam were detected in samples of bee-bread from the 24 control hives; CGA322704 was detected in only one control hive at 1.099 ng/g (with no detectable residues of thiamethoxam or CGA322704 in nectar which, together with the absence of thiamethoxam residues in bee-bread, would suggest robbing was not the source). By the end of the over-wintering period, residues in bee-bread from 17 samples available from control hives were low with only one hive with residues of 0.577 ng thiamethoxam/g, one hive with 1.058 ng thiamethoxam/g

and all others <LOD, with no detectable residues of CGA322704 in any hive. In nectar sampled from the 24 control hives at the end of dosing, residues of thiamethoxam were similarly low with only two hives with residues <LOQ, one with 1.785 ng/g and all others with residues <LOD, with no detectable residues of CGA322704. After winter residues of 0.577 ng thiamethoxam/g, and 0.664 ng thiamethoxam/g were each detected in one hive and the other 16 samples available all had residues <LOD, with no detectable residues of CGA322704 in any hive.

The residues of thiamethoxam and CGA322704 in bee-bread and nectar samples taken from the treated hives are shown in Fig. 1 for 2016 and for the 2014 and 2016 studies combined (see also Supplementary Data Table S3). All samples of bee-bread and nectar taken from treated colonies at the end of the dosing period contained only parent thiamethoxam residues (CGA2322704 residues were below LOQ). These data demonstrate that thiamethoxam residues were present not only in stored nectar but also found in significant concentrations in bee-bread after the end of the dosing period. In addition, the presence of detectable residues in the colonies that survived the winter confirms that the colonies were exposed via nectar and bee-bread during the over-wintering period.

3.2. Alternative forage sources

Although some of the pollen loads collected in the pollen traps potentially came from nearby agricultural fields, primarily corn (*Zea mays*), soybean (*Glycine max*) and tobacco (*Nicotiana*), these fields were not the main sources of forage for the bees. The majority of the pollen identified originated from non-crop sources: *Parthenocissus* spp. (Vitaceae), *Lagerstroemia*, *Plantago* and *Rhus*, were the predominant species in early summer; in summer *Rhus*, *Ambrosia*, *Medicago* and *Helianthus* were detected; in autumn the main source of forage were species of *Asteraceae*. Other species included *Trifolium* sp., *Xanthium* sp., *Tradescantia* sp. and *Eupatorium* sp.

Across all 60 pollen samples collected from the monitoring hives at each site only dicofol (7 samples positive with mean 45 ng/g, range: traces-59 ng/g), alachlor (5 samples positive with mean 80 ng/g, range: 65–93 ng/g) and thymol (23 samples positive with mean 62,760 ng/g, range: traces to 382,000 ng/g) were detected in multiple samples. Highest residues of thymol were from samples taken after *Varroa* treatments of hives in August and September. Carbendazim, pyridaben, metalaxyl and diflubenzuron were detected in single samples only at trace levels.

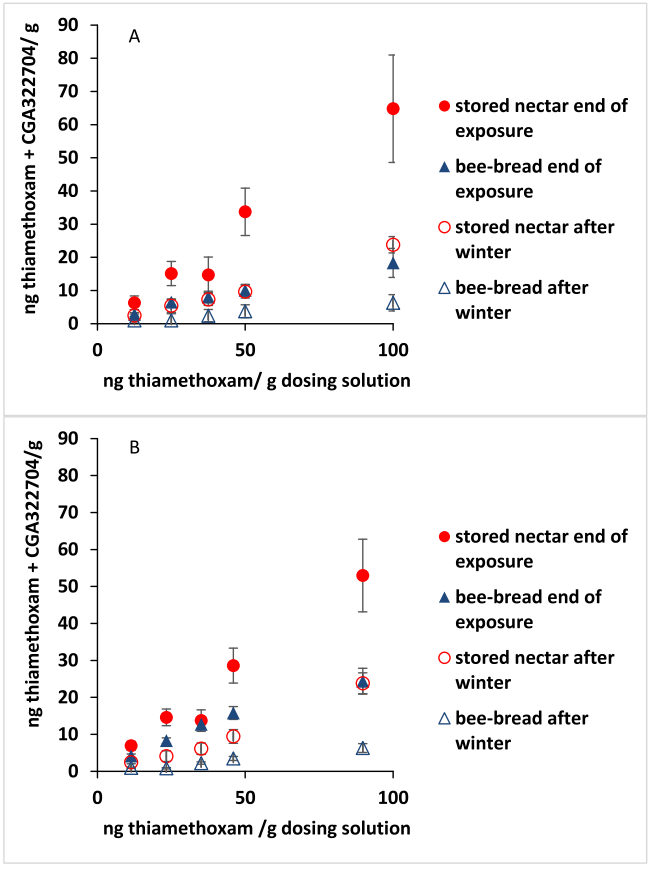

Fig. 1. Mean (±SE) residues of thiamethoxam and metabolite CGA322704 (clothianidin) in stored nectar and bee-bread A) from hives dosed at 12.5, 25, 37.5, 50 or 100 ng thiamethoxam/g sucrose in 2016; B) combined data from hives dosed in 2014 at 12.5, 25, 37.5, 50 or 100 ng thiamethoxam/L sucrose and 2016 (doses as A). X-axis reflects actual (measured) sucrose solution concentrations. Where residues were below the LOD or LOQ the LOD/2 or LOQ/2 respectively were assigned in calculating residues.

3.3. Pre-wintering data for 2014 and 2016

The detailed data for the 2016 study are provided in the Supplementary Data (for 2014 data see Overmyer et al. (2018)). Analysis of the combined data from the 2014 and 2016 studies (48 control colonies, 24 colonies per thiamethoxam treatment at the start of the dosing period, Fig. 2 and Supplementary Data Table S4) shows that, compared to control, the highest dose treatment group (100 ng thiamethoxam/g) has significantly fewer adult bees in the first assessment after the start of dosing (3 WAE) ($p = 0.003$), through to the last assessment before over-wintering (16 WAE) ($p = 0.002$). The 50 ng thiamethoxam/g treatment group also has significantly fewer adult bees compared to control at the end of dosing 6 WAE ($p = 0.002$) and 10 WAE ($p = 0.033$). Brood levels are similarly affected; compared to control, the highest dose treatment group (100 ng thiamethoxam/g) has significantly fewer total brood cell numbers from the first assessment after the start of exposure 3 WAE ($p < 0.001$) to 13 WAE ($p = 0.001$) driven by effects on numbers of eggs, larvae and pupae (Fig. 2). The 50 ng thiamethoxam/g treatment group also has significantly fewer total brood cells compared to control and at the end of the dosing period 6 WAE ($p = 0.008$) driven by effects on numbers of pupae. There is a significant reduction in pupal cell numbers compared to control for the 25 ng thiamethoxam/g dose group but only at 13 WAE ($p = 0.013$): Given that no effects on the overall levels of brood are observed in this treatment and this effect is not observed at the same late time-point post-exposure at dose levels of 37.5 and 50 ng thiamethoxam/g, this observation is likely an anomaly. As expected, all colonies began to reduce the numbers of bees and amount of brood produced in response to the approaching winter.

Analysis shows effects of exposure to thiamethoxam on the amount of stored bee-bread (pollen stores) during the dosing period (3 WAE and 6 WAE) in the 37.5 ng thiamethoxam/g ($p = 0.014$ and $p = 0.008$), 50 ng thiamethoxam/g ($p < 0.001$ and $p < 0.001$) and the 100 ng thiamethoxam/g ($p < 0.001$ and $p < 0.001$) treatment groups. After the end of the dosing period there are significant reductions in the 50 ng thiamethoxam/g ($p = 0.012$) and 100 ng thiamethoxam/g ($p < 0.001$) treatment groups at 10 WAE and these significant reductions in pollen stores continue in the 100 ng thiamethoxam/g treatment group to 13 WAE ($p < 0.001$) and 16 WAE ($p < 0.001$). There is significantly less nectar storage compared to control during the dosing period in the 12.5 ng thiamethoxam/g (3 WAE: $p = 0.007$, 6 WAE: $p = 0.015$) and 25 ng thiamethoxam/g (3 WAE: $p = 0.044$) dose groups. However, as there are no significant effects at higher dose levels during or after dosing, except at a single time point in the 100 ng thiamethoxam/g treatment group at 16 WAE, these effects are unlikely to be treatment related.

Changes in colony weight reflected the changes in numbers of bees, brood and pollen with significant effects only identified in the 100 ng thiamethoxam/g treatment (Fig. 2).

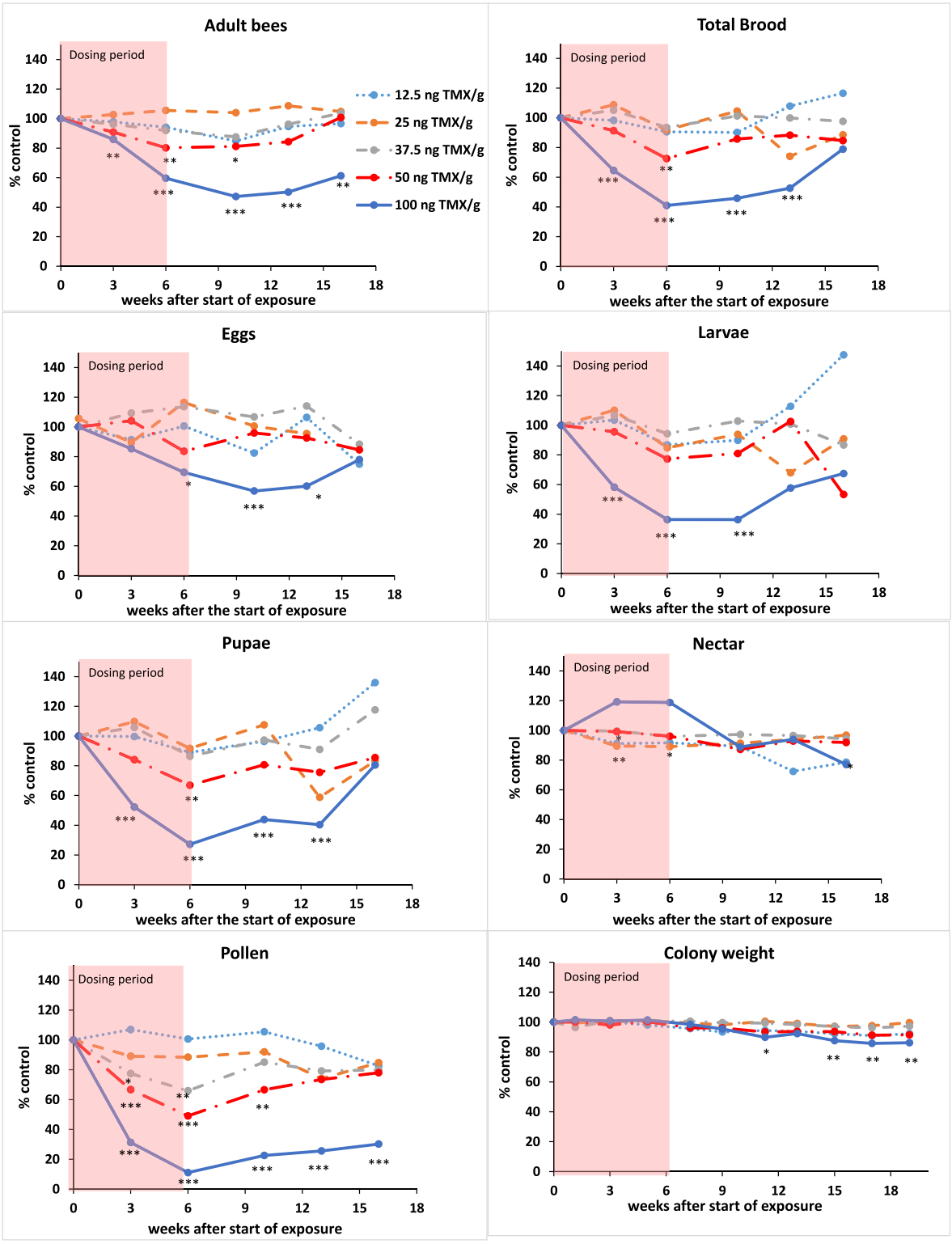

Fig. 2. Changes in the size of colonies relative to control (based on Least Mean Square data); combined data from studies in 2014 and 2016 from the start of dosing to last assessment before winter. Statistically significant results are identified below the data point $^{*}p \leq 0.05$, $^{**}p \leq 0.01$; $^{***}p \leq 0.001$.

3.4. Overwintering

In the 2016 study, three (25%) of the colonies fed with 100 ng thiamethoxam/g died before over-wintering. During the over-wintering period (November 2016–March 2017) two (8.3%) colonies in the control treatment, two (16.7%) colonies in the 25 ng thiamethoxam/g treatment and one (8.3%) colony in the 50 ng thiamethoxam/g treatment group were lost. An additional two colonies died by the April assessment (39 WAE), one in the control treatment (total 12.5%) and one in the 12.5 ng thiamethoxam/g treatment (total 8.3%). Of the 74 surviving colonies, 10 swarmed in the spring (35 WAE and 39 WAE): two in the control treatment, two in the

12.5 ng thiamethoxam/g treatment, three in the 25 ng thiamethoxam/g and three in the 50 ng thiamethoxam/g treatment (Supplementary Data Table S2). Swarming is generally accepted as an indication that the hives were building up normally and were healthy (Winston, 1987).

3.5. Pests and diseases

In assessing the levels of *Varroa* mites it is important not just to consider the reported number of mites per 100 adult bees but also the size of the colony, since any decrease in numbers of adult bees will result in an increase in concentration of the remaining phoretic mites (those present on adult bees). Therefore the change in total population of phoretic mites (calculated as mites per bee multiplied by the adult bee population) in the colonies from the 2016 study is shown in Fig. 3 (raw data are shown in the Supplementary Data Table S5). This shows a large increase in the phoretic mite population in the 100 ng thiamethoxam/g treatment in 2016 which may be an indirect effect of the significant reduction in brood by 6 weeks after the start of exposure, i.e. fewer suitable aged brood cells were available to the mites and thus the proportion of phoretic mites increased. *Nosema* levels were highly variable (Supplementary Data Table S6).

3.6. Residues in pollen and nectar from follow-on crops

Residues of thiamethoxam and CGA322704 in pollen and nectar collected from untreated follow-on crops planted in 2017 in the three field sites where thiamethoxam treated sugar beet was previously grown by farmers in 2016 are shown in Table 1. This shows residues in nectar were below the LOQ (0.5 ng thiamethoxam/g; 1 ng CGA322704/g) except in a single sample of oilseed rape nectar where a residue of 0.55 ng thiamethoxam/g was detected. Total residues (thiamethoxam + CGA322704) in pollen were more frequently detected above the LOQ than those in nectar although median values of thiamethoxam + CGA322704 were <LOQ in *Phacelia* and potato (anthers) at all sites, <LOQ–1.6 ng thiamethoxam + CGA322704/g in oilseed rape and <LOQ–1.8 ng thiamethoxam + CGA322704/g in the maize. All residues of thiamethoxam were below the LOQ in oilseed rape, potato (anthers) and *Phacelia* but up to 2.5 ng thiamethoxam/g was present in maize pollen (3 samples). Residues of CGA322704 were more frequently detected in pollen with up to 1.5 ng/g in oilseed rape (5 samples), 1.7 ng/g in potato (anthers) (2 samples), 6.3 ng/g in *Phacelia* (1 sample) and 1.3 ng/g in maize pollen (7 samples). This is in line with previous unpublished data in which residues of thiamethoxam and CGA322704 in *Phacelia* pollen were higher than those in oilseed rape grown in fields where crops had previously been grown from thiamethoxam treated seed (EFSA, 2018). Residues of thiamethoxam and CGA322704 detected in soil (pre-planting) collected from fields in which sugar-beet was grown in the previous season are shown in Table 2 and shows consistent residues over time within the field (from pre-planting to maize at flowering) but large variations between fields with lowest soil residues detected at the site with the highest previous use rate. There was no clear relationship between the level of detection of residues in soil and the subsequent residues in pollen as the highest residues in pollen were detected at the site with the lowest soil residues.

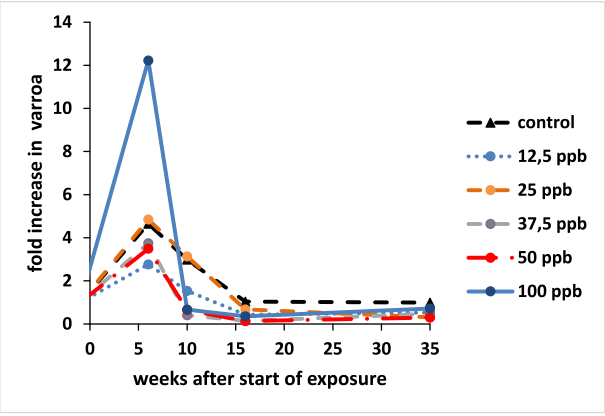

Fig. 3. Mean change in *Varroa* population (estimated by number of bees/hive and number of *Varroa*/100 bees) over time.

4. Discussion

The purpose of the honey bee colony feeding studies was to determine a threshold for adverse, colony-level, effects, defined as numbers of adult bees and brood, by feeding various concentrations of thiamethoxam-treated sucrose directly to the colonies over a 6-week period in a worst-case field setting with very limited alternative forage. This 6-week dosing period resulted in exposure of the colonies through the pre-winter and over-wintering period to residues in excess of those reported by a number of authors for nectar and pollen collected from flowering weeds around seed-treated crops (Botias et al., 2015; Tsvetkov et al., 2017) and thus also addresses this longer term exposure scenario. The colonies needed to forage for the pollen required for brood production throughout the studies and for additional nectar requirements after the end of the dosing period in an area with limited forage. Consistent exposure, and the presence of excess treated sucrose beyond that needed for colony survival, was demonstrated by the high residues in both bee-bread (processed pollen also containing nectar (Winston, 1987)) and nectar stored within the colonies in both studies at the end of the 6-week feeding period. Thus the bees within the colonies were exposed via both stored nectar and bee-bread, although primary exposure was via nectar as consumption of pollen is relatively low (Rortais et al., 2005). The colonies can be considered representative of normal, small to medium sized, "well-managed" colonies as they contained parasites (*Nosema* and low levels of *Varroa* mites at the start of the dosing period) and were also exposed to residues of other pesticides within the environment. Although virus levels were not specifically monitored, recent laboratory data (Coulon et al., 2018) suggests there is no interaction between thiamethoxam and one of the most common viruses, chronic bee paralysis virus, except at exposure levels (100–200 ng/mL) at which direct effects at the colony-level are expected. Analyses of the combined datasets from the two colony feeding studies indicates a clear dose-response; with consistent significant effects on colony-relevant endpoints following consumption at the 100 ng thiamethoxam/g sucrose, significant but transient effects on numbers of bees and brood levels at the 50 ng thiamethoxam/g treatment level and no significant effects on numbers of bees or brood in the 12.5, 25 or 37.5 ng thiamethoxam/g treatment groups.

In the 2014 study (Overmyer et al., 2018), most of the colonies across the thiamethoxam treatments and control did not survive overwintering due to insufficient feeding in late summer/early fall resulting in inadequate colony size (i.e., small winter cluster size) and food stores. In the 2016 study the over-winter loss of colonies in the control and thiamethoxam treatment levels up to 50 ng thiamethoxam/g (12.5%) was below the more widely reported average of 21.1% in the US in 2017/18 and the 10-year average of 28.4% (BeeInformed, 2017), whereas 25% of the 100 ng thiamethoxam/g treatment colonies were lost. The build-up of the colonies to swarming in the spring across all treatments up to and including the 50 ng thiamethoxam/g treatment also demonstrates the health of the remaining colonies. These data support the conclusions of the previous BeeHave modelled scenario in which loss of up to 20–25% of adult workers had no adverse long-term impact on the colony even when forage conditions were set as less than optimal (Thorbek et al., 2017): In the 100 ng thiamethoxam/g treatment there was a loss of up to 50% adult worker bees compared to control pre-winter, whereas in the 50 ng thiamethoxam/g treatment there were up to 20% fewer adult bees present at the end of the dosing

Table 1
Residues of thiamethoxam (TMX) and metabolite CGA322704 (clothianidin) in pollen and nectar sampled from untreated crops grown in fields where treated sugar-beet was grown in the previous season. Number of samples above LOQ isshown in superscript.

TMX product		Cruiser Force treated sugar-beet (spring 2016)			Cruiser Force treated sugar-beet (spring 2016)			Cruiser 600 FS treated sugar-beet (spring 2016)		
Application rate (g a.s./ha)		75			54			60		
Location		Groβ-Umstadt, Germany			Bergen, Germany			Remisbreite, Austria		
		Median pollen/nectar residues (range) (ng/g)								
		TMX	CGA322704	TMX + CGA322704	TMX	CGA322704	TMX + CGA322704	TMX	CGA322704	TMX + CGA322704
Oilseed rape	Pollen	<LOQ (<LOQ)	1.1 (<LOQ–1.5)[5]	1.6 (<LOQ–2.0)[5]	<LOQ (<LOQ)	<LOQ (<LOQ)	<LOQ (<LOQ)	ND	ND	ND
	Nectar	<LOQ (<LOQ)	<LOQ (<LOQ)	<LOQ (<LOQ)	<LOQ (<LOQ–0.55)[1]	<LOQ (<LOQ)	<LOQ (<LOQ–1.1)[1]	ND	ND	ND
Phacelia	Pollen	<LOQ (<LOQ)	<LOQ (<LOQ–6.3)[1]	<LOQ (<LOQ–6.8)[1]	<LOQ (<LOQ)	<LOQ (<LOQ)	<LOQ (<LOQ)	<LOQ (<LOQ)	<LOQ (<LOQ)	<LOQ (<LOQ)
	Nectar	<LOQ (<LOQ)	<LOQ (<LOQ)	<LOQ (<LOQ)	<LOQ (<LOQ)	<LOQ (<LOQ)	<LOQ (<LOQ)	<LOQ (<LOQ) (N = 8)	<LOQ (<LOQ) (N = 8)	<LOQ (<LOQ) N = 8
Maize	Pollen	<LOQ (<LOQ)	1.3 (<LOQ–2.5)[7]	1.8 (<LOQ–3.0)[7]	<LOQ (<LOQ)	<LOQ (<LOQ–2.6)[1]	<LOQ (<LOQ–3.1)[1]	<LOQ (<LOQ–2.5)[3]	<LOQ (<LOQ)	<LOQ (<LOQ–3.0)[3]
Potato	Pollen	ND	ND	ND	<LOQ (<LOQ)	<LOQ (<LOQ–1.7)[2]	<LOQ (<LOQ–2.2)[2]	<LOQ (<LOQ)	<LOQ (<LOQ)	<LOQ (<LOQ)

TMX = thiamethoxam.
n.d. = not determined; osr plants were not viable due to dry weather conditions; potato flowers were not available.
Thiamethoxam LOQ = 1.0 ng/g (pollen); 0.5 ng/g (nectar); CGA322704 LOQ = 1.0 ng/g (pollen and nectar); for residues <LOQ value replaced with 50% LOQ for calculation of total residues of TMX + CGA322704.

period (6 WAE) and at 10 WAE and no increased colony loss overwinter. The significant effect on levels of stored pollen during the dosing period in colonies dosed at 37.5 ng thiamethoxam/g sucrose and above is similar to reported reductions in pollen collection for colonies with pollen traps fitted and then fed thiamethoxam/clothianidin treated pollen (Sandrock et al., 2014), i.e. but was not reported for in-hive pollen levels in colonies with pollen traps fitted and fed imidacloprid-spiked pollen (Dively et al., 2015). In the 37.5 ng thiamethoxam/g treatment group the effect was reversed by 10 WAE but was more prolonged in the two highest treatments suggesting the effect may be inter-related with the observed effects of treatment on brood levels at these dose levels. Despite the dosing period equating to two brood cycles, adverse colony effects, i.e. those either directly or indirectly resulting in reductions in the numbers of bees or levels of brood, only persisted beyond dosing in the 100 ng thiamethoxam/g sucrose treatment group; significant effects on adult bees and brood at 50 ng thiamethoxam/g sucrose were transient recovering by 13 WAE, i.e. two brood cycles after the end of dosing. A colony development and survival NOEC (no observed effect concentration) of 37.5 ng thiamethoxam/g sucrose, NOAEC (no observed adverse effect concentration, where adverse relates to colony development and survival) of 50 ng thiamethoxam/g sucrose and LOEC (lowest observed effect concentration) of 100 ng thiamethoxam/g sucrose provide a robust basis to evaluate the potential risk to colony-relevant endpoints of residues of both thiamethoxam and its metabolite CGA322704 detected in pollen or nectar following treatment of bee-attractive crops with thiamethoxam. In this respect a colony-relevant endpoint for CGA322704/clothianidin is also required; a provisional endpoint of 19 ng clothianidin/kg has been published by the USEPA (EPA, 2017) which is in line with Solomon and Stephenson (2017). The USEPA (EPA, 2017) also proposed converting thiamethoxam to clothianidin equivalents. However, this assumes that internal concentrations of clothianidin converted from thiamethoxam in vivo reach

Table 2
Residues of thiamethoxam (TMX) and metabolite CGA322704 (clothianidin) in soil sampled from untreated crops grown in fields where treated sugar-beet was grown in the previous season.

TMX product	Cruiser Force treated sugar-beet (spring 2016)			Cruiser Force treated sugar-beet (spring 2016)			Cruiser 600 FS treated sugar-beet (spring 2016)		
Application rate (g a.s./ha)	75			54			60		
Location	Groβ-Umstadt, Germany			Bergen, Germany			Remisbreite, Austria		
	Soil residues (ng/g)								
	TMX	CGA322704	TMX + CGA322704	TMX	CGA322704	TMX + CGA322704	TMX	CGA322704	TMX + CGA322704
No crop, before drilling	<LOQ	1.9	2.4	1.1	5.4	6.5	7.1	9.9	17
Oilseed rape at flowering	<LOQ	3.0–4.0	3.5–4.5	<LOQ–3.5	7.1–11.0	7.4–14.5	3.9–4.8	14.0–16.0	18.1–20.8
Phacelia at flowering	<LOQ	2.1–2.6	2.6–3.1	1.7–2.5	6.0–7.2	8.1–9.7	2.6–6.7	8.3–9.5	10.9–16.2
Maize at emergence	<LOQ	1.7–2.4	2.2–2.9	1.3–2.9	4.4–6.9	6.7–9.0	4.1–10.0	8.7–10.0	12.8–20.0
Maize at flowering	<LOQ	1.8–2.7	2.3–3.2	1.6–2.3	4.4–5.9	6.0–8.2	3.1–4.7	9.4–10.0	13.0–14.1
Potato at flowering	ND	ND	ND	1.3–2.2	6.4–7.8	8.6–10.0	1.7–4.9	7.3–11.0	9.0–15.9

TMX = thiamethoxam.
n.d. = not determined.
Thiamethoxam LOQ 1.0 ng/g and CGA322704 LOQ 0.1 ng/g.
For residues <LOQ value replaced with 50% LOQ for calculation of total residues of TMX + CGA322704.

those equivalent internal concentrations following externally applied doses (Coulon et al., 2018). If this was the case then the endpoints of thiamethoxam and clothianidin would be equivalent and following oral exposure they clearly are not (EPA, 2017). Therefore, the most appropriate method for colony level assessment is to use a toxic unit approach for residues in pollen and nectar in which the amount of CGA322704 and thiamethoxam TUs are based on the differences in colony level NOAEL, currently 19 ng/g and 37.5 ng/g respectively, i.e. 1 TU CGA322704 is equivalent to 0.5 TU thiamethoxam. Thus no conversion of the feeding solutions is required but data on residues in pollen and nectar and the agreed colony-feeding study endpoint for clothianidin are required.

The approach of directly feeding colonies with sucrose treated with a range of thiamethoxam concentrations to determine a colony level NOEC addresses the current challenges in the conduct of bee field studies in Europe: The EFSA guidance document (EFSA, 2013) requires that the exposure assessment goal has to be matched in every field study, i.e. residues in pollen and nectar are greater than or equal to the 90th percentile predicted exposure levels in every replicate (EFSA, 2013; EFSA, 2018). It also provides additional support for the lack of effects reported in field studies following exposure of colonies to flowering bee-attractive crops such as maize (pollen only), sunflowers and winter oilseed rape grown from seed treated with thiamethoxam (Hernando et al., 2018; Pilling et al., 2013; Woodcock et al., 2017) where residues in pollen/bee-bread and nectar were at least an order of magnitude lower than the NOEC and NOAEC in this study. However, these results also suggest effect levels are far higher than those reported in other studies. Explanations may include the mode of dosing, feeding treated pollen patties requires pollen traps to be fitted which reduce pollen availability within the colony which may exacerbate any effects (Sandrock et al., 2014; Tsvetkov et al., 2017). As sister queens were not used, but queens from a single source were used, major differences in sensitivity between strains could not be addressed (Sandrock et al., 2014) although published data for thiamethoxam suggests *A m. ligustica* are more sensitive than *A m carnica* (Laurino et al., 2013; Rinkevich et al., 2015). The colonies used in this study were small-medium sized (3000–22,000 adult bees across the 2014 and 2016 studies). Although this study could not be used to assess differences based on colony size, as this is compounded with any site differences, small colonies are likely to have less resilience to any factors which may have impacts on the numbers of adult bees and brood (Thorbek et al., 2017; Wu-Smart and Spivak, 2016) and as shown in 2014 are also less successful in overwintering.

The thiamethoxam and CGA322704 residues in stored nectar sampled from the colonies can also be compared with those recently reported for surveys of honey samples (Mitchell et al., 2017; Woodcock et al., 2018). Combining data from these two studies showed >65% of samples had residues below the LOQ cited in the respective paper and the 90th percentile was 0.6 ng thiamethoxam + clothianidin/g honey. This 90th percentile value is at least an order of magnitude below those identified following treatment at the NOEC of 37.5 ng thiamethoxam/g sucrose (mean end of exposure value of 13.7 ± 2.9 ng thiamethoxam + CGA322704/g nectar and mean post-winter stored nectar residue of 6.1 ± 1.5 ng thiamethoxam + CGA322704/g). Even accounting for any differences between thiamethoxam and clothianidin in the colony level NOEL it is highly unlikely that adverse effects would have been observed in the colonies from which the honey was sourced.

The results of the feeding study can also be compared with those reported by (Wood et al., 2018) who fed 80 nM thiamethoxam (~20 ng thiamethoxam/g) to package bee colonies over a 6-week period and reported a 30% effect on colony weight gain (not statistically significant) and 21% statistically significant effect on bee cluster size visually assessed as filling inter-frame spaces (but not number of adult bees measured by digital analysis) after 9–12 weeks. The data were generated from 12 control and 9 treated colonies and the authors raised concerns that the study did not have the power to detect effects on numbers of bees and brood. The comparable concentration used in this study is the 25 ng thiamethoxam/g rate, where 48 control and 24 treated colonies were used, which showed no significant effect on colony weight compared to controls while the 10% difference observed in the 100 ng thiamethoxam/g treatment group was significant ($p < 0.05$), giving an indication of power. As in the Wood et al. (2018) study, there were no significant effects on the number of adult bees and brood in the 25 ng thiamethoxam/g treatment, however, in this study an observed 20% difference for the 50 ng thiamethoxam/g treatment was highly significant ($p < 0.01$), giving an indication of power.

As the colony feeding study data demonstrated a clear dose-response effect, these data can also be used to validate decision point "trigger" values in regulatory risk assessments. These "trigger" values at the first tier screening level aim to clearly separate those pesticide uses which require further evaluation from those which pose no risk to honey bees. Therefore trigger values are based on predicted "worst-case" exposure together with laboratory effects data (either mortality, e.g. LD50 or no mortality based endpoints e.g. NOEL) and include additional safety factors. A low rate of false positives (uses which indicate risk but are demonstrated in higher-tier testing, e.g. semi-field and field studies, to be of low risk) at the first tier screening level is acceptable since the schemes are clearly aimed at avoiding false negatives. However, a high false positive failure rate questions the setting of the trigger value and potentially mis-directs large amounts of resources to the unnecessary conduct and evaluation of higher-tier data (EFSA, 2013; USEPA, 2014). Honeybee mortality incident data have previously been used in Europe to validate the acute risk assessment approach including the first tier trigger value (Thompson and Thorbahn, 2009). However, honeybee mortality incidents are clearly not appropriate for validating chronic tier 1 risk assessment outcomes. In using the colony feeding study data to validate the current tier 1 chronic adult honeybee risk assessment approach the scenario can be considered highly conservative; alternative forage was limited and the treatment was fed directly into the colony while requiring the colony to continue foraging for pollen during exposure and for both pollen and nectar after the 6-week dosing period for the remaining 8 months of the study, in the same study area.

There has been particular concern in Europe on the detection of residues of neonicotinoids in pollen and nectar sampled from follow-on crops or flowering weeds at the edges of cropped fields. The residue data reported in this study are from pollen and nectar collected from bee-attractive crops planted in fields where treated sugar-beet was grown in the previous season. The data on soil residues in this study are comparable with the results of a larger survey of 50 fields conducted by the European sugar-beet industry (CIBE, 2018) and therefore can be considered representative. The data demonstrate the very low residues present in pollen and nectar with mean residues below the LOQ (0.5 ng/g nectar, 1 ng/g pollen); mean pollen and nectar residues being most relevant for chronic exposure scenarios. These data can be directly compared with laboratory toxicity data in a refined (exposure) first tier (Tier 1) assessment. For thiamethoxam, the chronic toxicity to adult bees is a daily dose LD_{50} of 4 ng/bee/day (LC50: 190 ng thiamethoxam/g diet) and daily dose NOEL of 2.45 ng/bee/day (NOEC: 117 ng thiamethoxam/g diet) (Overmyer et al., 2018). For honey bee larvae, the 22-day larval toxicity is a NOEC of 102 ng thiamethoxam/g diet (NOEL: 15.7 ng/larva/development period) (Overmyer et al., 2018). At these levels the proposed EFSA first tier chronic risk assessment (EFSA, 2013) clearly fails for adult forager honey bees (mean consumption of 533 mg nectar = 0.267 ng thiamethoxam results in an Exposure Toxicity Ratio (ETR) of 0.067 based on the daily dose LD_{50}, exceeding the "trigger" of 0.03) although no concerns are raised for larvae (consuming 396 mg nectar, 2 mg pollen = 0.2 ng thiamethoxam results in an ETR of 0.012 based on the NOEL, below the "trigger" of 0.2) or in the US EPA chronic risk assessment (USEPA, 2014) (RQ adult bees =

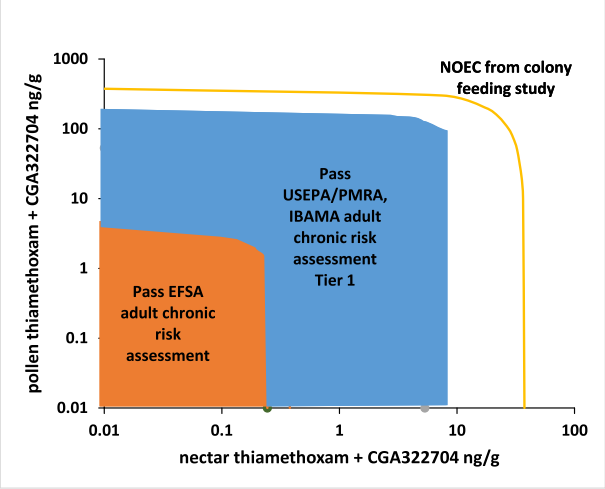

Fig. 4. Comparison of the residues in pollen and nectar which currently trigger higher tier testing in the EFSA (EU) (EFSA, 2013) and USEPA/PMRA (USA/Canada) (USEPA, 2014), IBAMA (Brazil) (IBAMA, 2017) based on honey bee first tier adult chronic risk assessments for in-hive bees and forager bees with the NOEC of 37.5 ng thiamethoxam/g from the colony feeding study. LOQ based on 50% LOQ for thiamethoxam + 50% LOQ for CGA322704 is 0.75 ng/g for nectar and 1.0 ng/g for pollen.

0.06, RQ larvae = 0.004; both well below the "trigger" of 1 based on the NOELs).

Comparison of the EFSA risk assessment outcomes with the colony feeding study NOEC of 37.5 ng thiamethoxam/g nectar shows that the "trigger" is failed in the first tier of the EFSA risk assessment at nectar residues over 100 fold below the colony level NOEC, and also below the analytical LOQ (Fig. 4). This can be explained in part by the assumption in the EFSA risk assessment that an adult bee consumes over 5 times its bodyweight of nectar (assumed 15% sugar content) per day while other risk assessment schemes, e.g. USEPA, assume consumption of 3 times bodyweight of nectar (assumed 30% sugar content). However, a major contributor to this over-conservatism is the incorrect default assumption in the proposed EFSA scheme that the only dose at which there is 0% mortality, compared with control in the laboratory adult chronic toxicity study, is at 0 ng/g nectar. In contrast, the chronic adult laboratory bee data for thiamethoxam demonstrates mortality at or below control levels at doses up to 1.77 ng/bee/day (Overmyer et al., 2018). Correcting this assumption in the EFSA tier 1 chronic risk assessment (adjusting the slope of the assumed dose-response) results in combined residues in nectar and pollen of up to 3.45 ng thiamethoxam + CGA322704/g passing the first tier chronic adult honey bee risk assessment. This still provides an order of magnitude of safety compared with the colony-level NOEC of 37.5 ng thiamethoxam/g. Further improvements could be achieved by adjusting sugar content of nectar, etc. for more realistic values. Making relative these small changes to the EFSA risk assessment would also provide greater alignment with the results of honey bee risk assessments elsewhere (USEPA, 2014; IBAMA, 2017).

These data, collated from two separate feeding studies, have shown that continuous exposure of honey bee colonies fed for 6 weeks with thiamethoxam concentrations up to and including 37.5 ng thiamethoxam/g sucrose results in no significant effects on the number of adult bees and brood. Exposure to 50 ng thiamethoxam/g sucrose results in transient effects but no long-term adverse effects to the colony. Feeding with 100 ng thiamethoxam/g sucrose for 6 weeks resulted in significant effects on the colonies before winter and subsequently on over-wintering success. Comparison of these data with recently published global and national honey survey results (Mitchell et al., 2017; Woodcock et al., 2018), data from pollen and nectar from seed-treated crops (Hernando et al., 2018; Jiang et al., 2018; Pilling et al., 2013; Woodcock et al., 2017) and from follow-on crops within this study has also demonstrated that these reported residues are at least an order of magnitude below those resulting in impacts on colony-relevant endpoints.

Conflict of interest

This work was funded by Syngenta LLC which manufactures and sells products containing thiamethoxam. The authors are either employed by Syngenta or by Eurofins AgroScience Services who performed the data generation (in compliance with GLP) under contract.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2018.11.003.

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