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Thiamethoxam Honey Bee Colony Feeding Study: Linking Effects at the Level of the Individual to Those at the Colony Level

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Abstract: Neonicotinoid insecticides have been used globally on a wide range of crops through seed treatment as well as soil and foliar applications and have been increasingly studied in relation to the potential risk to bees because of their detection in pollen and nectar of bee-attractive crops. The present article reports the results of laboratory studies (10-d adult and 22-d larval toxicity studies assessing the chronic toxicity of thiamethoxam to adult honey bees and larvae, respectively) and a colony feeding study, with 6 wk of exposure in an area with limited alternative forage, to provide a prewintering colony-level endpoint. The endpoints following exposure of individuals in the laboratory (10-d adult chronic no-observed-effect concentration [NOEC] for mortality 117 μg thiamethoxam/kg sucrose solution, 141 μg thiamethoxam/L sucrose solution; 22-d larval chronic NOEC 102 μg thiamethoxam/kg diet) are compared with those generated at the colony level, which incorporates sublethal effects (no-observed-adverse-effect concentration [NOAEC] 50 μg thiamethoxam/L sucrose solution, 43 μg thiamethoxam/kg sucrose solution). The data for sucrose-fed honey bee colonies support the lack of effects identified in previous colony-level field studies with thiamethoxam. However, unlike these field studies demonstrating no effects, colony feeding study data also provide a threshold level of exposure likely to result in adverse effects on the colony in the absence of alternative forage, and a basis by which to evaluate the potential risk of thiamethoxam residues detected in pollen, nectar, or water following treatment of bee-attractive crops. *Environ Toxicol Chem* 2017;9999:1–13. © 2017 SETAC

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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). Because of their widespread use and systemic properties, this class of insecticides has been increasingly studied in relation to the potential risk to bees as a result of their detection in pollen and nectar of bee-attractive crops (Pisa et al. 2014). Recent discussions on the effects of insecticides on pollinators have centered on the effects of neonicotinoids on individual bees, with an increasing number of studies reporting sublethal effects (Pisa et al. 2014; Intergovernmental Science-Policy Platform on Biodiversity and Ecosystem Services 2016). Nitroguanidine neonicotinoid insecticides are inherently toxic to honey bees (the acute oral median lethal dose of thiamethoxam is 5 ng/bee), and there have been assertions that toxic effects are cumulative following chronic exposure (Tennekes 2011). However, currently it is not possible to link sublethal effects at the individual level with impacts at the colony level, limiting the utility of such laboratory studies in risk assessments for these insecticides under real-use conditions (Godfray et al. 2015); this is particularly the case because the aim of such risk assessments is to address protection goals including pollination services, hive product production, and biodiversity, which are dependent on colony-level assessment endpoints (US Environmental Protection Agency 2014). Available studies on the effects of neonicotinoids at the colony level are limited, primarily because of the resources required to conduct these highly complex studies. The few field exposure studies that have been reported were designed to understand the potential impact on honey bee colonies when applied to crops following

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typical agronomic practices (Pilling et al. 2013; Cutler et al. 2014). However, results from these field studies are crop- and use-specific (primarily seed treatment) and might not be applicable to other crops and uses where exposure to honey bees could be different.

Colony feeding studies have been developed to directly assess the insect growth-regulating properties of insecticides (Oomen et al. 1992), but because these studies were designed to determine mode of action rather than effect levels, high concentrations of active ingredients were typically used (often at tank concentrations applied in the field rather than the levels likely to be returned to the hive by foraging honey bees). More recently there has been regulatory interest (e.g., US Environmental Protection Agency [USEPA], European Food Safety Authority) in conducting colony feeding studies to determine the pesticide level in nectar substitute (sucrose solution) which leads to colony-level effects, thereby allowing for comparison with residues detected in pollen and nectar from treated crops. To date, only a few published studies have been conducted in this manner (i.e., directly exposing honey bee colonies to neonicotinoid-treated feed at high concentrations), and these have been limited to imidacloprid (Faucon et al. 2005; Dively et al. 2015), which has been demonstrated to have antifeeding properties in some bee species (Thompson et al. 2015) and therefore may not be representative of other neonicotinoids.

Both laboratory and field (colony feeding) studies, reported herein, were conducted for regulatory purposes, including for the USEPA as part of the registration review process for thiamethoxam (US Environmental Protection Agency 2011). Laboratory studies (10-d adult and 22-d larval toxicity studies) were conducted to assess the chronic toxicity of thiamethoxam to adult honey bees and larvae, respectively, according to draft Organisation for Economic Co-operation and Development (OECD) guidelines. Colony feeding studies were conducted to provide a robust colony-level endpoint for comparison with residues in pollen and nectar detected following applications in bee-attractive crops. The laboratory and colony feeding studies investigate potential effects of thiamethoxam at scales of organization from the individual to the colony level, respectively. The present article compares the effects following exposure of individuals in the laboratory with colony-level effects, which incorporate sublethal effects, and assesses these endpoints in terms of actual exposure to environmentally relevant concentrations in the field.

METHODS

Laboratory toxicity studies

Honey bees, *Apis mellifera carnica* (Hymenoptera: Apidae), for the chronic adult and larval studies, were sourced from an apiary owned by Eurofins Agroscience Services (no antibiotics, anti-*Varroa* treatments, pesticides, and so on, had been used in the hives for at least 1 mo prior to this test). One hive was used for the chronic adult study; 3 separate hives were used in the chronic larval study. Thiamethoxam was supplied as a technical standard (99.5%) by Syngenta, and the toxic reference, dimethoate, was purchased as the formulation Perfekthion (analyzed content 420 g/L).

Chronic adult toxicity. The experiment was conducted as a dose–response study with a duration of 10 d and starting with 1- to 2-d-old adult honey bees (Organisation for Economic Co-operation and Development 2016). Up to 2 d prior to test start, brood combs containing capped cells (expected to hatch on the same day) were taken from a single colony and transferred into a growth chamber maintained at 31.3 to 33.5 °C and 50 to 70% relative humidity. One day prior to the test, 0- to 1-d-old emerged bees were transferred to test cages (10 bees/cage) and kept in the same environmental conditions. Test cages were made of stainless steel (base 8×4 cm, height 6 cm), with the front side of the cages equipped with a transparent pane to enable observation and the bottom of the cages made of perforated steel, to ensure sufficient air supply, and lined with filter paper. A 50% (w/v) aqueous sucrose solution was used as food ad libitum during acclimatization. Any moribund or dead bees were replaced before the start of exposure with bees from the same source and age.

The test was comprised of 2 control groups, 5 test item (thiamethoxam) groups, and 1 toxic reference item group (dimethoate). Each treatment group consisted of 4 replicate cages, each containing 10 bees. The exposure period lasted 10 d.

Untreated 50% (w/v) sucrose solution containing 0.5% acetone and the dimethoate (toxic reference item) stock solution were stored under cool and dark conditions in the refrigerator ($\sim 6 \pm 2$ °C) for a maximum period of 96 h. A stock solution of thiamethoxam was prepared in acetone every day of the study. The feeding solutions for all treatments were freshly prepared every day either by diluting the stock solution directly in 50% (w/v) aqueous sucrose solution or from higher concentrated solutions with 50% (w/v) aqueous sucrose solution containing 0.5% acetone to ensure that all solutions contained the same concentration of acetone. This resulted in feeding solution test concentrations of 36.2, 65.2, 117.3, 211.1, and 380 μg thiamethoxam/kg (43.4, 78.2, 140.8, 253.3, and 456 μg thiamethoxam/L). The toxic reference (0.9 mg dimethoate/kg 50% [w/v] sucrose) was prepared from the stock solution daily.

The feeding solutions were offered to the test organisms in each test unit in feeders consisting of a 5-mL plastic syringe. The bees in one cage shared the feeding solution and thus received similar doses (by trophallaxis). Approximately 3 to 4 mL of feeding solution was offered to the bees daily, and this volume guaranteed ad libitum feeding during each 24-h feeding interval. Freshly prepared feeding solution replaced the feeding solution of the previous day by changing feeders for preweighed replacements. The amount of feeding solution consumed was determined by weighing the feeders before and after feeding.

To determine feeding solution loss from the feeders by evaporation under the conditions of the study, 8 test units without bees but with full food syringes containing approximately 3 to 4 mL of pure 50% (w/v) aqueous sucrose solution or 50% (w/v) aqueous sucrose solution containing 0.5% acetone (v/v) were placed in the climatic chamber. The syringes of 8 test

units were filled and weighed each day to determine daily loss through evaporation.

Mortality and behavioral abnormalities were recorded every 24 h (± 2 h) after feeding solution introduction. Behavioral abnormalities were recorded in the control and thiamethoxam treatment groups according to the following categories: affected (bees still upright and attempting to walk but showing corrected signs of reduced coordination), apathy (bees show only low or delayed reactions to stimulation, e.g., light or blowing; sitting motionless in the unit or able to walk but not correctly), cramps (bees contracting abdomen or entire body), moribund (bees cannot walk and show only very feeble movements of legs and antennae, only weak response to stimulation, e.g., light or blowing; bees may recover but usually die), or vomiting. The consumption of feeding solution per bee per day was calculated by dividing the total daily consumption per replicate by the number of bees alive at the start of the respective feeding interval. For each treatment group, the mean consumption of feeding solution per bee per day was calculated by averaging the replicate values. A mean value of evaporation per day was determined, and the daily food consumption (of each replicate) of the control(s), test item, and reference item treatments was corrected by the mean value of the corresponding day.

Samples (10 mL) of the feeding solution from the solvent control and all the thiamethoxam test item groups were taken every day directly after preparation. Samples were frozen (–18 °C) within 45 min after sampling until analysis.

The step-down Cochran-Armitage test (one-sided greater, $\alpha = 0.05$) was used to evaluate whether there were significant differences between the mortality data of the control and the test item treatment group and to determine the no-observed-effect concentration (NOEC) and no-observed-effect daily dose based on mortality. Statistical calculations were made using the statistical program ToxRat Professional 3.2.1.

Chronic larval study. The experiment was conducted as a dose–response test with a duration of 22 d according to draft OECD Guidance (Organisation for Economic Co-operation and Development 2014). It covered a developmental phase from grafting of first-instar larvae on day 1 to the final assessment of adult emergence on day 22. First-instar honey bee larvae (L1), originating from 3 different colonies, detailed in *Laboratory toxicity studies*, were used for the experiment.

The study was comprised of one control group (C); 6 thiamethoxam groups (T1–T6) with concentrations of 0.102, 0.203, 0.406, 0.813, 1.63, and 3.25 mg thiamethoxam/kg diet, equivalent to cumulative doses of 0.0157, 0.0313, 0.0625, 0.125, 0.251, and 0.501 μg thiamethoxam/larva; and one reference item group with a concentration of 48.0 mg dimethoate/kg diet, equivalent to a cumulative dose of 7.39 μg dimethoate/larva.

For preparation of the larval diet, a fresh commercial royal jelly was used. The batch of royal jelly was certified organic; the absence of antibiotics, pesticides, and heavy metals was confirmed by a multiresidue analysis. Three diets were prepared and fed to the larvae on different days of the test. Larvae were fed 20 μL of diet A (50% weight of royal jelly + 50% weight of an aqueous solution, containing 2% weight of yeast extract, 12% weight of glucose, and 12% weight of fructose) on day 1; 20 μL of diet B (50% weight of royal jelly + 50% weight of an aqueous solution, containing 3% weight of yeast extract, 15% weight of glucose, and 15% weight of fructose) on day 3; and 30, 40, and 50 μL of diet C (50% weight of royal jelly + 50% weight of an aqueous solution, containing 4% weight of yeast extract, 18% weight of glucose, and 18% weight of fructose) on days 4, 5, and 6, respectively, resulting in a cumulative feeding volume of 140 μL diet/larva. Larvae were not fed on day 2. The larval diet was freshly prepared in advance and divided into aliquots that were stored frozen (≤ –18 °C) until use. Just before feeding, the diet was thawed in an incubator and the test item and reference item solutions were added to the diet using a micropipette. The diet was then homogenized by shaking. Because the larval diet was prepared in adjusted concentration considering an absence of 10% (v/v) water, the lacking volume of water was added as 10% (v/v) test item solution. For the control group 10% (v/v) of the final diet volume was added as autoclaved, deionized water. For the reference item group 10% (v/v) of the final diet volume was added as reference item solution. Larvae were exposed to the test item or reference item starting on day 3 and ending on day 6.

Care was taken to avoid touching and drowning the larvae during feeding. Treatments started with the control group, followed by the test item groups (with increasing concentrations) and lastly the reference item group. A new pipette tip was used for each treatment group. Samples of the larval diet from each thiamethoxam group and the control group were taken directly from the prepared diet on each dosing day (days 3, 4, 5, and 6) for chemical analysis. Samples were stored frozen (≤–18 °C) within 1 h after sampling until analysis.

All plates were placed in the same incubator with forced air circulation at 34 to 35 °C. On day 1, the plates of the control, test item, and reference item groups were placed into a desiccator containing a dish with a saturated potassium sulfate solution to keep a water-saturated atmosphere. On day 8, the plates of the control, test item, and reference item groups were placed into a desiccator containing a dish with a saturated sodium chloride solution to maintain humidity. On day 15, a lid was placed on each plate and transferred from the desiccator into an incubator containing a tray of deionized water to maintain humidity.

From days 1 until 22, the air temperature and relative humidity were recorded in intervals of 15 min with calibrated data loggers placed inside each desiccator. The temperature was maintained between 34 and 35 °C, and not below 23 or above 40 °C, for more than 30 min once every 24 h (i.e., during feeding). From days 1 to 8, larvae were held at 95 ± 5% relative humidity, from days 8 to 15 at a relative humidity of 80 ± 5%, and on days 15 to 22 at a relative humidity of 50 ± 10%.

Mortality during the larval phase (days 1–8) was assessed before feeding on days 4 through 6 as well as on days 7 and 8 when no food was provided. Larvae were recorded as dead if no respiration (movement of spiracles) was observed. Mortalities during the pupation phase (days 8–22) were assessed on days 15 and 22. The number of emerged bees was assessed on day 22. If necessary, mortality was assessed with the assistance of a

stereomicroscope; dead larvae and pupae were removed when identified.

Fisher's exact test with Bonferroni correction (one-sided greater, $\alpha = 0.05$) was used to evaluate whether there was a significant difference between the mortality data of the test item groups and the control group on day 22.

Analytical methods for the laboratory studies. The analytical method selected for the determination of thiamethoxam in adult chronic feeding solutions was validated with regard to recovery, linearity of detector response, repeatability, specificity, limit of quantification (LOQ), and limit of detection (LOD). At the analytical laboratory, samples were thawed, ultrasonicated for 20 min, and shaken using a Vortex mixer. A 5-mL aliquot was transferred into a 100-mL Schott bottle; 45 mL of methanol/water (1:1, v/v) + 0.1% formic acid were added and shaken for 20 min using a flatbed shaker. If necessary, samples were further diluted with matrix blank dilution after shaking the Schott bottle prior to analysis by high-performance liquid chromatography–tandem mass spectrometry (HPLC-MS/MS). The test item dilution (120 mg/L) was used for fortification of 600 μg/L recovery samples in 50% (w/v) aqueous sucrose solution containing 0.5% acetone. The test item stock dilution (4 mg/L) was used for fortification of 20 μg/L recovery samples in 50% (w/v) aqueous sucrose solution containing 0.5% acetone. Analysis by HPLC/MS-MS used the Agilent 1290 Infinity HPLC system Applied Biosystems API 5000 (Analyst 1.5.2 [rev. 1.6.2 for processing]) with Waters Atlantis dC18 100 A, 75 × 4.6 mm i.d., 3 μm mean particle size (no. 186001333), with a 4-mm guard column; the injection volume was 50 μL, temperature was 40 °C, mobile phase A was water + 0.1% formic acid, and mobile phase B was methanol + 0.1% formic acid. Within the sequence, the detector linearity was confirmed over the relevant calibration range by constructing a calibration function of peak area versus concentration within the range from 0.2 to 30 μg/L. Injections of sample extracts were interspersed with injections of quality control standards after a maximum of 5 samples to verify the detector response and to adjust the calculated concentration. Recovery was 101 ± 2% (relative standard deviation [SD]) at 20 μg/L and 93 ± 4% (relative SD) at 600 μg/L.

For analysis of the larval diet, approximately 500 mg of diet was transferred into a 15-mL plastic tube and weighed. For preparation of matrix blank extract, untreated larval diet (diet C) was used. Ten milliliters of methanol:water (1:1, v/v) + 0.1% formic acid adjusted to the sample weight were added, and the samples were homogenized for 10 min on a flatbed shaker. Samples were then centrifuged at 4000 rpm for 5 min. After phase separation, samples were diluted with demineralized water. Solid-phase extraction (SPE) procedures were implemented using Oasis™ HLB cartridges (60 mg, 3-mL size), for each sample. For phase equilibration, 3 mL methanol were added and drawn through a vacuum to the level of the top frit at a rate of approximately 2 mL/min. Column eluates were discarded. The SPE cartridges were not allowed to become dry. Demineralized water (3 mL) was then added to the cartridges and drawn through a vacuum to the level of the top frit at a rate of approximately 2 mL/min. Column eluates were discarded. The SPE cartridges were not allowed to become dry.

Samples (5 mL each) were transferred to the SPE cartridge and drawn through a vacuum to the level of the top frit at a rate of approximately 2 mL/min. Residues of thiamethoxam were retained on the SPE cartridge. Demineralized water (1 mL) was added to the tubes that contained the sample extracts. Tubes were rinsed and transferred to the corresponding SPE cartridge. The water was drawn through a vacuum to the level of the top frit at a rate of approximately 2 mL/min. Column eluates were discarded. Any remaining droplets of water adhering to the inside of the SPE cartridge walls were removed with an adsorbent tissue and dried under high vacuum for approximately 20 min.

After drying, 2 mL of hexane were added to the SPE cartridge and drawn through a vacuum at a rate of approximately 2 mL/min. Column eluates were discarded. A high vacuum was applied for 20 to 30 s to remove any remaining solvent from the SPE cartridges. Glass tubes (10 mL) were placed under each port as required in the manifold rack. Six milliliters of acetonitrile were added to the SPE cartridges and drawn through a vacuum at a rate of approximately 2 mL/min. Column eluates were collected in the 10-mL glass tubes. A high vacuum was applied for 2 to 3 s to remove any remaining solvent from the SPE cartridges. Residues of thiamethoxam were eluted in this fraction.

Acetonitrile fractions were evaporated to dryness under a stream of clean, dry air. An elevated temperature of 40 °C was used to aid the process. Residues were dissolved in 1 mL methanol/water (1:9, v/v) and ultrasonicated thoroughly. Samples were then analyzed by HPLC-MS/MS as described previously. Recovery was 89 ± 8% (relative SD) at 0.05 mg/kg and 88 ± 12% (relative SD) at 4.5 mg/kg.

For each treatment group the cumulative dose per larva (micrograms of thiamethoxam per larva per developmental period) was calculated based on the given test item concentration (milligrams of thiamethoxam per kilogram diet), the cumulative feeding volume per larva, and the density of the diet (1.1 g/cm^3).

Colony feeding study

The study area in central North Carolina, USA (on rural lands between the cities of Durham and Greensboro; Orange, Caswell, and Alamance Counties), is comprised primarily of forested and pasture land with some small tracts of crops such as tobacco, corn, and soybeans (Supplemental Data, Figure S1). Consequently, the potential for exposure of honey bees to pesticides outside of this test area was relatively low. During the 6-wk exposure phase, temperature (daily minimum/maximum), relative air humidity (daily minimum/maximum), and rainfall were recorded from weather stations at 2 apiary sites (0.16–12.07 km from the apiaries).

Honey bees (*Apis mellifera ligustica*) were purchased as packages from a commercial bee supplier (J J's Honey). *Apis mellifera ligustica* is the subspecies of honey bee that most commercial beekeepers in the southeastern United States use for their operations because it is better adapted to the warmer

climatic conditions typically encountered in this region. The bees were installed in new hive boxes (10-frame Langstroth boxes) at the beginning of May 2014 with a new queen (supplied by the same source) introduced into each colony. The cohort of over 100 colonies was assessed twice (colony condition assessments [CCA1 and CCA2]; Imdorf et al. 1987; Imdorf and Gerig 1999), and of these, 96 colonies were selected for the study based on general health, with no visible symptoms of *Varroa* or *Nosema* or other bee diseases, as well as having all stages of brood, a queen, and some food stores. At the third colony assessment in July (CCA3), which was just prior to the start of the exposure (dosing) period, each colony consisted of 1 or 2 brood boxes (10 frames in each box), 3689 to 22 081 adult bees, 3 to 12 brood combs with all brood stages (except 2 hives in the 25 μg/L treatment which contained no brood and 1 in the 37.5 μg/L group which contained one frame of brood as a result of swarming prior to CCA3), and 4 to 16 combs containing nectar, honey, and bee bread (stored, processed pollen with honey added).

Eight hives were selected for each of the 12 study apiary sites (A–L; Supplemental Data, Figures S1 and S2): 7 hives for biological assessments (2 untreated and 1 for each of the 5 thiamethoxam treatment levels) and 1 monitoring hive that served to assess what the bees were foraging on in the landscape and potential pesticide exposure from other sources. Assignment of study apiaries to the apiary locations was done randomly. Each hive was allocated to 1 of 12 study apiary sites stratified by brood strength of the colony: apiary A contained the strongest group of hives, and apiary L contained the weakest group of hives. Hives were moved from the holding yards to the study apiary site locations (all apiaries were at least 2 km apart) at the end of June 2014 (Supplemental Data, Figure S2). Each hive within an apiary was spatially separated from other hives by at least 9 m, and hives were arranged in a semicircular pattern, facing east to west, with 38-m spacing between the 2 hives at the ends of the semicircle to minimize drifting and deter robbing. Hives were arranged on pallets to raise them above ground level and had an empty deep super on top, between the lid and the inner cover, to allow dark space to contain the sugar solution feeder. This allowed the feeder to be placed on the inner cover so that only bees from that hive had access and exposure to direct sunlight was prevented.

Each colony allocated to biological assessment was provided untreated sucrose (50% [w/w] in tap water) or sucrose (50% [w/w] in tap water) containing 12.5 to 100 μg thiamethoxam/L sucrose solution (technical thiamethoxam [98.9% purity]; Syngenta). A stock solution of 0.050 g of technical thiamethoxam dissolved in approximately 20 mL of acetone was prepared and diluted with tap water to 1 L. Sugar solutions were prepared (50% [w/w]) by diluting 4.25 (T1), 8.5 (T2), 12.75 (T3), 17 (T4), or 34 (T5) mL of stock thiamethoxam solution in 17 L of sugar solution to create dosing solutions containing 12.5 (T1), 25 (T2), 37.5 (T3), 50 (T4), or 100 (T5) μg thiamethoxam/L sucrose solution (w/v).

A Boardman sugar solution feeder was used to administer the thiamethoxam sugar solution during the exposure period. Untreated or treated sucrose (1 L) was fed twice per week for 4 wk and 1.5 L untreated or treated sucrose twice per week for the final 2 wk (because of increased colony strength). The exposure period started during the first week of July 2014 and ended in mid-August 2014. At each renewal, freshly prepared sugar solution was provided to each colony and any solution remaining from the previous feeding was removed and measured. In 2014 to 2015 the surviving colonies were fed 1 L of a 2:1 sugar:water solution on a weekly to fortnightly basis from late autumn (end of October) through the winter (through mid-April).

Dosing solution concentrations were determined from samples taken at the time of dosing, and solution stability was determined under hive conditions by collection of samples after placement of dosing solution, sealed in 15-mL Falcon tubes, in monitoring hives at each apiary site 1 and 5 wk after initiation of the exposure period (they were not available to the colony). These stability samples remained in the hives until the next dosing solution change and feeding event, after which they were collected and stored frozen.

Colony condition assessments were conducted to observe the overall colony performance from before the start of exposure on 2 to 6 July 2014 (CCA3); during exposure on 28 to 31 July 2014 (CCA4); immediately after exposure on 20 to 28 August 2014 (CCA5); 3 times prior to winter on 17 to 23 September 2014 (CCA6), 6 to 10 October 2014 (CCA7), and 27 to 29 October 2014 (CCA8); and twice after overwintering on 31 March 2015 (CCA9) and on 28 to 29 April 2015 (CCA10). Because of the size of the study, each CCA period in 2014 took 2 or more d to complete; in 2015 CCAs took 1 to 2 d because the size and number of colonies needing to be assessed were reduced as a result of overwintering loss. During the assessments, colonies were also checked for visible symptoms of disease or pests, such as *Nosema*, foulbrood, *Varroa* mites, or small hive beetle.

Samples of adult honey bees were collected from each biological hive at 3 time points during the study to assess *Varroa* infestation: once before start of exposure, once after exposure and before overwintering, and once after overwintering. Bees collected from each hive were stored frozen until further processing. Bees were washed in alcohol to remove mites, to calculate the number of mites per 100 bees.

At CCA9 honey bee samples were also used to assess the number of *Nosema* spores in the remaining hives. Thirty worker bees were dissected, and the abdomen was removed. Abdomens were placed in a mortar with 10 mL distilled water and ground, followed by the addition of 2 aliquots (10 mL each) of distilled water. The number of spores in this suspension was counted with a hemocytometer under a microscope (×400 magnification), and the number of spores per bee was calculated.

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. Because of the number of colonies that needed to be assessed, the apiaries were split between 2 experts for the CCAs. Each expert only assessed hives in the 6 designated apiaries, to reduce any potential

added variability as a result of the different experts conducting the CCAs. The total number of cells per frame is 3440 (based on 400 cells/cm^2 and 860 cm^2/comb side); therefore, to calculate total number of cells of brood or food stores the percentage coverage was multiplied by 3440 for one side of a frame. The number of adult bees was also estimated by area of the frame covered using digital imaging, and the total bee population was estimated by assuming a density of 130 bees/100 cm^2. Digital photographs were transferred to a computer for analysis using software Irfanview (Ver 4.38) and Mousetron (Blacksun Software [2001–2015], Ver 5.0, 9.1, and 10.0). The data were transferred to Excel (2010, Ver 14.0.6 and 14.0.7) and SAS (Ver 9.3) for statistical analysis calculations.

The weight of each hive was monitored continuously using a digital balance (B-ware™ Beehive Monitoring System from Solutionbee). Balances recorded the weight and temperature at each hive on an hourly basis. The balances were installed under the hives 1 wk after CCA3 and remained until the final CCA (CCA10) after overwintering. During the week of CCA3 the hive weights were recorded manually. Several balances failed sporadically during the study as a result of technical problems, and during these periods hive weights were recorded manually with a calibrated balance.

Samples of bee bread and nectar were collected at CCA3 from all hives before the exposure phase, 6 wk after the start of exposure during CCA5 and after overwintering at CCA10 for analysis of thiamethoxam and its major metabolite CGA322704 (clothianidin). Samples of bee bread were collected from at least 3 different areas of frames per colony using pollen corers and pooled per colony. Nectar samples were collected with a single-use plastic spoon or other suitable clean tool. A sample consisted of nectar collected from at least 3 different frames (if available) per colony.

Samples of pollen and nectar were collected from the monitoring hive at each apiary preexposure and 1, 3, 5, 6, 10, 13, and 16 wk after start of exposure of the treated hives.

Pollen traps were placed at the entrances of all monitoring hives and activated at 8 time points over the course of the study. Pollen traps remained activated (pollen grids closed) for approximately 24 to 48 h. The timing of pollen trap activation was adapted to pollen availability. Pollen samples were split into 2 subsamples: one for pollen identification and one for residue analysis (pesticide screening; analyzed for 174 active ingredients using multiresidue methods). Because external pollen availability varied, pollen amounts collected from each hive 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; samples for multiresidue pesticide screening were shipped from EPL to the US Department of Agriculture laboratory (Gastonia, NC). Samples for pollen identification were shipped to the Niedersächsisches Landesamt für Verbraucherschutz und Lebensmittelsicherheit (LAVES), Institut für Bienenkunde.

Analytical methods for the feeding study. Pollen analysis for screening of a wide range of pesticides was undertaken by the US Department of Agriculture using the methodology described in Mullin et al. (2010).

Sucrose used for treatments and stability assessments, bee bread, and nectar were analyzed for thiamethoxam and metabolite CGA322704 by EPL, Bio-analytical Services. Methods were verified prior to analysis of samples (all recoveries were within 80–120% and relative SD <20%). Spiked sample recoveries performed in parallel with sample analysis were 74.9 to 114% and 74.2 to 109% for thiamethoxam and CGA322704, respectively, in bee bread and 70.4 to 118% and 70.4 to 119% for thiamethoxam and CGA322704, respectively, in nectar and sucrose.

Nectar and bee bread samples (100 mg) were extracted in methanol:0.2% formic acid in deionized water (50:50) and diluted with water, and stable isotope-labeled internal standards were added. Sample extracts were purified by SPE (Oasis HLB) and analyzed with triple-quadrupole LC-MS/MS (Agilent 1290; column Kinetex XB-C18 100A, mobile phase 0.1 formic acid in water: 0.1% formic acid in methanol, with ABSciex q-trap 6500 or Triple Quad 6500) with limits of quantification (LOQ) of 0.5 μg/L for nectar and 1 μg/kg for bee bread.

Pollen source identification. Distilled water was added to the pollen sampled from the pollen traps (1:5). For complete dispersion and homogenization the solution was stirred with a magnetic stirrer for approximately 1 h. A 15-μL aliquot of the homogenized water–pollen solution was transferred onto a microscope slide, 30 μL of distilled water were added, and the aliquot was homogenized again with a spatula and spread over an area of 22 × 22 mm. After drying on a warming plate at 40 °C, embedding took place with a drop of mounting medium (Kaiser's glycerine jelly), which was added with a pipette under a 22 × 22 mm coverslip.

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 to calculate their proportion in the total pollen sample.

After determining the proportion of the different pollen species in the first 100 grains, the next 100 grains were counted. This was repeated 5 times for each sample. If the results from the 5 counts were consistent, then no further analysis of the sample was undertaken.

Data Evaluation. The effects of thiamethoxam on honey bee colony health were evaluated by comparing the results from test item treatment hives to control hives: condition of the colonies (strength, visually and digitally assessed) and development of the bee brood and food stores based on CCA data; hive weight data; and residue data in the untreated and treated samples.

Although most CCAs were conducted over multiple days, for summary statistics, the first day was used to characterize any given CCA.

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).

Statistics for the colony feeding study. The statistical software program SAS (Ver 9.3) was used for the statistical analysis of brood and hive weight data as well as number of bees. For the predosing phase data, all statistical tests were done in a 2-tailed approach, whereas for the data assessed after application, one-tailed (lower) tests were conducted.

An analysis of covariance (ANCOVA) was performed on the Box-Cox transformed data for the data generated at the CCAs. To eliminate factors with possible impact on the data other than effects of treatment, the different apiaries as well as the last values of each observed subject were used as covariates for ANCOVA models. Three models were tested: ANCOVA with apiaries (blocks) as covariates, ANCOVA with apiaries and pretreatment values as covariates, and a one-way analysis of variance (ANOVA). The decision on which parameter was used for ANCOVA or if a simple ANOVA was conducted instead of the ANCOVA was made based on the significance of the impact of the parameter for the analysis. Impacts of covariates on the model were analyzed using significance tests (in particular, *F* tests) to determine whether the pretreatment values or apiary influenced the posttreatment values of each parameter. If the covariate was found to be significant, ANCOVA was selected, whereas if the covariate was found to be nonsignificant, ANOVA was selected. For each assessed subject, the pooled estimate of residual error variance obtained from the selected form of analysis (ANOVA or ANCOVA) was used to compare each treatment to the control using a 2-sided Dunnett's *t* test at the 5% significance level. If an ANCOVA was selected, the transformed means (and therefore the detransformed means) were adjusted for the effect of the covariate. Adjusting the means involves removing all differences between the treatment groups that can be accounted for by the covariate.

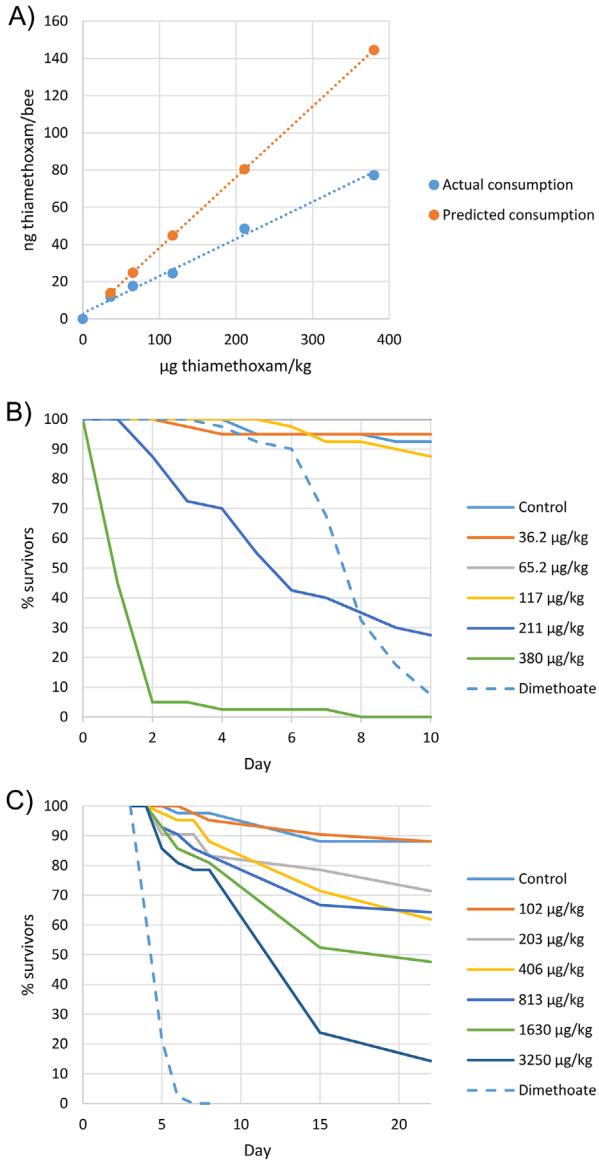

FIGURE 1: Exposure and effects of thiamethoxam on adult honey bees and larvae under laboratory conditions. Comparison of actual consumed dose with predicted (based on control consumption in the same study) by adult bees in a 10-d chronic toxicity study (**A**), resulting mortality of adult bees over time in the chronic toxicity study (**B**), and mortality of larvae fed with treated diet over time in a 22-d chronic toxicity study (**C**).

RESULTS

Laboratory studies

Chronic adult toxicity. The actual concentrations of thiamethoxam in all the feeding (sucrose) solutions were in the range of 83 to 119% of the nominal concentrations. In both control groups (with and without 0.5% acetone) 7.5% mortality was observed at the final assessment on day 10. Mortality by day 10 in the dimethoate toxic reference cages was 92.5%, and therefore, based on control and reference mortality, the study was considered valid. After 10 d of continuous exposure, the NOEC for mortality was 117 µg thiamethoxam/kg (141 µg thiamethoxam/L) sucrose solution, and the median lethal concentration (LC50) was 190 µg thiamethoxam/kg (95% confidence interval 173–204 µg thiamethoxam/kg) sucrose solution. The overall mean daily consumption of sucrose solution (i.e., the average consumption/bee over 10 d) decreased with increasing concentration of thiamethoxam such that after 10 d of continuous exposure, consumption was 40% lower in the highest treatment group than the control group (Figure 1); the median lethal daily dose and no-observed-effect daily dose were determined to be 0.00433 and 0.00245 µg thiamethoxam/bee, respectively. No behavioral effects were observed at 36.2 and 65.2 µg thiamethoxam/kg (43 and 78 µg thiamethoxam/L) sucrose solution, and only single bees were recorded as affected in the 117 µg thiamethoxam/kg (141 µg thiamethoxam/L) sucrose solution treatment; higher numbers of affected and moribund bees were recorded in the 211 and 380 µg thiamethoxam/kg (253 and 456 µg thiamethoxam/L) sucrose solution treatment groups.

Chronic larval toxicity. The study was considered valid because by day 8 larval mortality was 100% in the toxic reference item group and by day 22 the adult emergence rate

in the control group was 88.1%; the measured test item concentrations in the larval diet were within ± 20% of nominal. Thiamethoxam caused a statistically significant decrease in adult emergence in the 5 highest test item groups (0.203–3.25 mg thiamethoxam/kg diet; Fisher's exact test with Bonferroni correction, one-sided greater, $\alpha = 0.05$). Therefore, the NOEC was 102 μg thiamethoxam/kg diet, and the LC50 was 1.22 mg thiamethoxam/kg diet (95% confidence interval 0.579–6.16 mg thiamethoxam/kg diet).

Colony feeding study

The first 2 assessments (CCA1 and CCA2) were conducted solely for assessment of colony strength for the selection of the hives for the study and grouping by colony strength, and therefore, these will not be considered further. During the 6-wk exposure period, environmental conditions were within expected ranges for the locality with temperatures ranging from 14 to 36 °C and a total of 211 mm of rainfall accumulated (112 mm during July and 99 mm in the first 2 wk of August). During the following 2 mo, until the start of overwintering (end of October), temperatures ranged from 2 to 35 °C and a total of 84 mm of rainfall accumulated (81 mm during September and 3 mm during October).

There were no significant differences in *Varroa* (highest mite load was found in August 2014, mean range 1.5–2.6 mites per 100 bees) or *Nosema* (mean number of spores/bee ranged 750 000 [25 μg/L]–3 966 667 [37.5 μg/L]) levels between surviving colonies from treatment groups and control at the end of the winter.

Volume of sucrose consumed. The concentration of the test item in the sucrose solution remained stable (within 20% nominal) at the 2 assessment points during the study. The mean consumption of sucrose by colonies during the 6-wk exposure period is shown in Figure 2 and ranged from 49% (a colony in the 100 μg/L treatment group) to 100% of the total 14 L provided per hive. As shown in Figure 2, the sucrose solution was consumed completely in most hives in all treatments except the 100 μg/L treatment.

Residues in hive matrices. The residues of thiamethoxam in bee bread and nectar samples taken from the treated hives are shown in Figure 3. With the exception of a single bee bread sample in the 37.5 μg/L treatment group at CCA5 (after the end of feeding) where the thiamethoxam metabolite CGA322704 was detected at levels above the LOQ (1.36 μg/L), only parent thiamethoxam residues above the LOQ were detected in any bee bread or nectar samples taken during the treatment exposure period. These data demonstrate that thiamethoxam residues were present not only in stored nectar but also in significant concentrations in bee bread soon after the end of the exposure period. In addition, the lower levels detected in the colonies that survived over winter suggest that the treated sucrose was consumed by the colonies via nectar and bee bread.

Alternative forage sources. Although some of the identified pollen types potentially came from nearby agricultural fields, primarily corn (*Zea mays*) and tobacco (*Nicotiana*), these fields were not the main sources of forage for the bees. The majority of the pollen identified from pollen trap samples originated from noncrop sources: *Chenopodium* spp., *Plantago*, *Rhus*, *Ambrosia*, *Helianthus*, and Asteraceae-type, with lower preference for plants such as *Parthenocissus*, *Z. mays*, and *Lagerstroemia*. There were seasonal variations observed; in early summer *Parthenocissus*, *Z. mays*, *Lagerstroemia*, *Chenopodium*, *Plantago*, and *Rhus* were the predominant plants, whereas in summer mostly pollen of *Rhus*, *Ambrosia*, and *Helianthus* was detected. In fall, the bees foraged mainly on Asteraceae.

Pollen samples were collected 8 times from the monitoring hives at each site for assessment of potential contaminant exposure from outside sources. Across all samples the following residues were detected: acephate (1 sample, 1600 μg/Kg), carbaryl (1 sample, 214 μg/Kg), carbendazim (5 samples, traces 1300 μg/Kg), imidacloprid (2 samples, 4.3 and 6.1 μg/Kg), methamidophos (1 sample, 109 μg/Kg), thiamethoxam (2

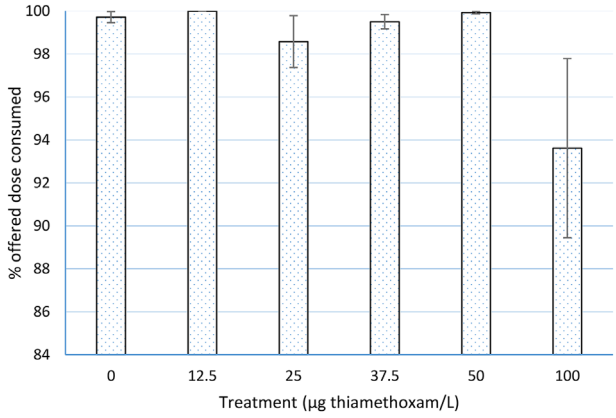

FIGURE 2: Mean (± standard error) percent offered dose consumed of untreated and thiamethoxam-treated sucrose by honey bee colonies over the 6-wk feeding period during the colony feeding study. Total volume of sucrose solution provided to each colony was 14 L.

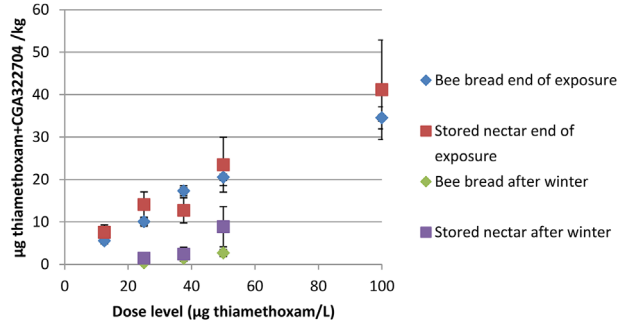

FIGURE 3: Residues of thiamethoxam and the major metabolite, CGA322704 (clothianidin), detected in bee bread and nectar sampled from treated colonies after the end of the exposure period and in surviving colonies after overwintering in the colony feeding study. None of the colonies from the 12.5 or 100 μg thiamethoxam/L treatment groups survived over the winter.

samples, 8.8 and 11.3 μg/Kg), and thymol (23 samples, 405–12300 μg/Kg, during periods of *Varroa* treatment).

Colony size. The control group and all test item treatment groups except the 100 μg/L treatment group demonstrated the same pattern in numbers of adult bees from preexposure to immediately before overwintering (CCA5; Figure 4), with fluctuations in the mean number of honey bees per colony likely reflecting the weather conditions at the time of assessment and seasonally changing food supply. The numbers of adult bees is driven by the amount of brood present in each colony (Figure 4). The brood nest (total number of brood cells) increased in the control, 12.5, 25, and 37.5 μg/L treatment groups, whereas the size of the brood nest in the 50 and 100 μg/L treatment groups was significantly reduced after the start of treatment feeding. As expected, all colonies began to reduce the number of brood produced in response to the approaching winter (after CCA5) with slight fluctuations in the brood nest size regarded as normal.

The size of the brood nest was further assessed by brood type: eggs, larvae, and pupae (Table 1). Of the endpoints assessed in the CCAs, egg cell counts were the most variable. In the control and treatment colonies, except for the 100 μg/L treatment, egg cell counts were consistent until CCA5, with numbers dropping from CCA5 to CCA8. Egg cell counts at the 100 μg/L treatment level were significantly different from the control at CCA5 and CCA6 (ANCOVA, one-sided, $p < 0.05$, pretreatment-adjusted). Although egg cell counts at the 12.5 μg/L treatment level were significantly different from the control at a single time point (CCA6), the lack of effect on egg cell counts at the 37.5 and 50 μg/L treatment levels or a statistically significant reduction in any other parameter at 12.5 μg/L suggests that the difference was not treatment-related. Larval cell counts (open brood) were also consistent until CCA6, again in all except the 100 μg/L treatment group, which contained significantly lower numbers of larval cells at both CCA5 and CCA6 (ANCOVA, one-sided, $p < 0.05$, pretreatment-adjusted). At the last CCA before overwintering (CCA8) colonies of all treatments groups had a similar proportion of larval cells. In the control and treatment groups up to 37.5 μg/L, the number of pupal cells (capped brood) increased from CCA3 until CCA5 and dropped steeply afterward until CCA8 (before overwintering). The colonies in the 50 and 100 μg/L treatment groups showed significant decline in pupal cell numbers after the start of feeding (Table 1), at the 50 μg/L treatment level at CCA4 and CCA5, and at the 100 μg/L treatment level at CCA4, CCA5, and CCA6 (ANCOVA, one-sided, $p < 0.05$, pretreatment-adjusted). Pupal cell counts at the 37.5 μg/L treatment level were significantly different from the control only at CCA5 (ANCOVA, one-sided, $p < 0.05$, pretreatment-adjusted).

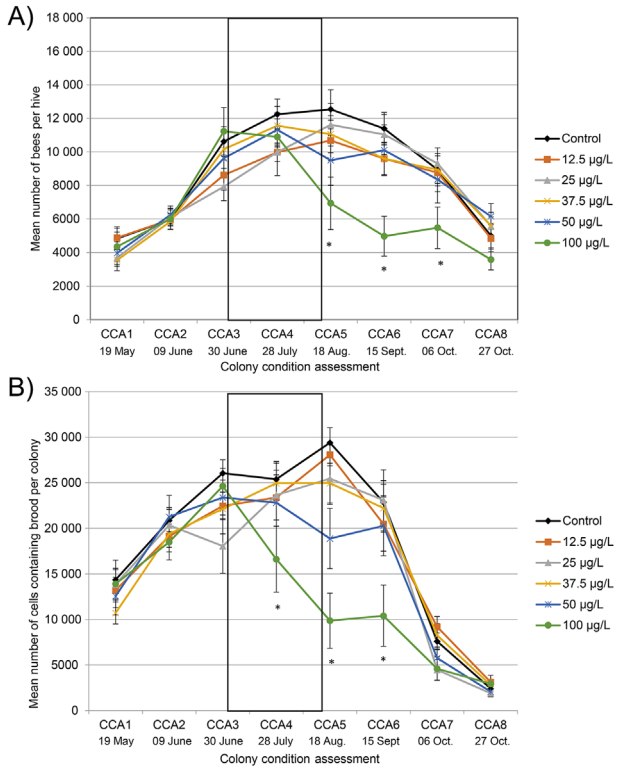

FIGURE 4: Mean (± standard error) numbers of adult honey bees (**A**) and brood (**B**) present in the colonies before, during, and after dosing with thiamethoxam in sucrose at different treatment levels in the colony feeding study. Boxes on the graphs between colony condition assessment (CCA) 3 and CCA5 identify the 6-wk treatment feeding period. * = significantly different from control ($p < 0.05$ analysis of covariance, pretreatment-adjusted).

The amount of stored bee bread steadily decreased during exposure between CCA3 and CCA4, increased by the September assessment (CCA5), and increased again until CCA7 in all treatment groups except the 100 μg/L treatment group (Table 1). The proportion of bee bread was significantly lower at CCA4 in treatment groups of 25, 50, and 100 μg/L (ANCOVA, one-sided, $p < 0.5$, pretreatment-adjusted). At CCA5, bee bread stores were significantly lower than the control for the 25 and 100 μg/L treatments, but by the prewintering assessment (CCA8) the bee bread stores of all colonies were on a similar level (ANCOVA, one-sided, $p < 0.5$, pretreatment-adjusted).

Nectar stores increased in all treatments except the 100 μg/L treatment until CCA5, dropped strongly until CCA7, and increased again until CCA8 (Table 1). Prior to winter the mean nectar stores were at a similar level in all treatment groups except the 100 μg/L treatment group. No statistically significant differences between the treatments were detected prior to wintering, and no dose-response trend was evident in hive weights, which also strongly reflected bee bread and nectar stores.

Overall, although significant effects in various endpoints were observed at the 50, 37.5, and 25 μg/L treatment levels, these effects were transient and did not result in significant reduction in hive strength (number of adults or total brood) over the course of the study. Based on these results, the no-observable-adverse-effect concentration (NOAEC) for the present study is considered to be 50 μg thiamethoxam/L sucrose solution (43 μg thiamethoxam/kg).

Overwintering. During the overwintering period, several consecutive days of below-normal low temperatures, with extended periods below freezing, occurred in early February 2015. This, coupled with the late start of maintenance feeding

TABLE 1: Detailed data for colony condition assessments (CCA) for pretreatment (CCA3) to prewinter (CCA8) during the colony feeding study with *Apis mellifera* (L.)[a]

Treatment	Stage	CCA3 30 June	CCA4 28 July	CCA5 18 August	CCA6 15 September	CCA7 6 October	CCA8 27 October
Control	Eggs	6063 ± 606	6020 ± 501	5289 ± 528	5743 ± 710	2587 ± 329	665 ± 135
	Larvae	5920 ± 446	6034 ± 582	6722 ± 474	5796 ± 662	1735 ± 301	500 ± 106
	Pupae	14 068 ± 639	13 330 ± 1400	17 386 ± 954	11 315 ± 1460	3275 ± 488	1243 ± 217
	Adults	10 616 ± 882	12 248 ± 902	12 542 ± 1169	11 390 ± 966	8941 ± 965	4999 ± 728
	Nectar/ honey	15 358 ± 1513	16 756 ± 1500	18 612 ± 2144	20 087 ± 2690	13 356 ± 1866	18 990 ± 2099
	Bee bread	3755 ± 536	3218 ± 439	4608 ± 526	3806 ± 485	3814 ± 555	2510 ± 366
T1 (12.5 μg/L)	Eggs	5375 ± 422	5361 ± 542	6163 ± 1209	3406 ± 679*	2374 ± 319	688 ± 188
	Larvae	4902 ± 477	6221 ± 855	6135 ± 480	5573 ± 753	2270 ± 587	533 ± 137
	Pupae	12 183 ± 904	11 796 ± 1536	15 767 ± 758	11 472 ± 1893	4541 ± 729	1892 ± 494
	Adults	8618 ± 906	9979 ± 544	10 684 ± 1209	9594 ± 946	8767 ± 1131	4843 ± 803
	Nectar/ honey	12 398 ± 1845	12 628 ± 1911	12 728 ± 2123	19 212 ± 2634	13 382 ± 1915	19 384 ± 3435
	Bee bread	3096 ± 605	3153 ± 705	3641 ± 791	3732 ± 619	3801 ± 607	2442 ± 471
T2 (25 μg/L)	Eggs	4114 ± 842	6077 ± 932	4959 ± 485	5457 ± 649	1908 ± 368	719 ± 129
	Larvae	3440 ± 688	5834 ± 900	6049 ± 788	5973 ± 1012	813 ± 225	297 ± 57**
	Pupae	10 506 ± 1807	11 739 ± 1610	14 448 ± 1917	11 649 ± 2055	1736 ± 790	876 ± 235
	Adults	7928 ± 835	9993 ± 1401	11 619 ± 1272	11 038 ± 1193	9309 ± 929	5575 ± 553
	Nectar/ honey	14 176 ± 1201	15 322 ± 1399	14 620 ± 2424	17 403 ± 2473	13 823 ± 2228	19 827 ± 2296
	Bee bread	3139 ± 529	1577 ± 417*	2594 ± 556*	2768 ± 448	2095 ± 495	1923 ± 372
T3 (37.5 μg/L)	Eggs	4802 ± 740	6034 ± 563	5676 ± 556	5395 ± 561	3143 ± 375	625 ± 130
	Larvae	4945 ± 771	6307 ± 606	6364 ± 748	5848 ± 871	1829 ± 354	594 ± 161
	Pupae	12 370 ± 1470	12 613 ± 1420	12 916 ± 1472*	10 977 ± 1880	3284 ± 1004	1579 ± 372
	Adults	10 163 ± 1143	11 565 ± 11557	11 058 ± 1109	9597 ± 980	8942 ± 828	5595 ± 796
	Nectar/ honey	17 530 ± 2151	17 960 ± 1882	19 061 ± 2855	22 235 ± 2922	15 683 ± 3006	20 703 ± 3040
	Bee bread	4128 ± 802	2895 ± 801	3768 ± 652	3409 ± 527	3331 ± 536	2846 ± 536
T4 (50 μg/L)	Eggs	5590 ± 571	6364 ± 781	4443 ± 842	5194 ± 981	2098 ± 378	516 ± 188
	Larvae	4988 ± 746	5877 ± 779	4357 ± 875	5074 ± 894	1651 ± 433	191 ± 116
	Pupae	12 800 ± 1011	10 564 ± 1509*	10 076 ± 1826*	10 010 ± 1996	2012 ± 588	1357 ± 336
	Adults	9621 ± 991	11 324 ± 1021	9500 ± 1488	10 100 ± 1508	8358 ± 1404	6152 ± 767
	Nectar/ honey	14 391 ± 1415	16 182 ± 1048	17 831 ± 1772	20 296 ± 2922	15 669 ± 1995	19 646 ± 2079
	Bee bread	2967 ± 589	1863 ± 494*	2867 ± 596	2941 ± 571	2993 ± 514	2542 ± 460
T5 (100 μg/L)	Eggs	6536 ± 694	5504 ± 836	2938 ± 470*	2546 ± 541*	1892 ± 438	1081 ± 29
	Larvae	4945 ± 760	4085 ± 1081	2666 ± 781*	2632 ± 909*	1474 ± 539	442 ± 175
	Pupae	13 144 ± 984	7023 ± 2153*	4257 ± 2113*	5229 ± 2044*	1229 ± 460	1401 ± 306
	Adults	11 229 ± 1417	10 898 ± 1071	6934 ± 1564*	4972 ± 1185*	5475 ± 1238*	3578 ± 619
	Nectar/ honey	15 824 ± 1581	22 346 ± 1321	19 230 ± 2923	15 971 ± 2055	14 448 ± 2614	14 448 ± 2614
	Bee bread	4171 ± 957	1390 ± 635*	889 ± 481*	1152 ± 685	1990 ± 623	2113 ± 854

[a]Values are mean ± standard error. For eggs, larvae, pupae, nectar/honey, and bee bread, values represent the number of frame cells occupied per colony. For adults, values represent number of individuals per colony.
*$p < 0.05$ analysis of covariance (ANCOVA), pretreatment-adjusted.
**$p < 0.05$ ANCOVA, unadjusted.

after the dosing phase, which resulted in bee populations and food stores in the colonies being low going into winter, resulted in many control and treated colonies not surviving the overwintering period. By the end of April 2015, from the 12 sites with a combined 84 hives, 82% of hives were considered dead (control 79.2% died; 12.5 and 100 μg/L, 100% died; 37.5 μg/L, 83.3% died; 25 and 50 μg/L 66.7% died). Data for CCA9 and CCA10 are shown in Supplemental Data, Table S1.

DISCUSSION

The present data indicate adverse effects of thiamethoxam at residue levels >100 μg/L (100 μg/kg) for individual bees/larvae under laboratory conditions and at residues >50 μg/L (43 μg/kg) for the colony. These NOAECs for the individual and for the colony are an order of magnitude above thiamethoxam residues reported in nectar and pollen following seed treatment uses, which have been the focus of many of the field studies (Blacquiere et al. 2012; Pilling et al. 2013; Godfray et al. 2014, 2015). There have been suggestions that the effects of neonicotinoids on individual bees are cumulative (Tennekes 2011). This can be investigated for thiamethoxam using the adult honey bee 10-d laboratory data by determining the total actual consumed doses where close to 50% mortality was observed (Supplemental Data, Table S2). A dose of 5.8 ng/bee supplied in a single day and a total dose of 28 ng consumed over the course of 6 d (i.e., 4.6 ng/bee/d) resulted in the same level of individual mortality (55–57.5%). This can be compared with a total dose of 24.5 ng/bee over a 10-d period (i.e., 2.45 ng/bee/d), which resulted in only 12.5% mortality. Because the dose per bee per day and not the total dose are key in determining the level of mortality observed, the effects are not cumulative. This result is

in accordance with reports that thiamethoxam is rapidly metabolized, and residues of the bee toxic metabolite CGA322704 are also not accumulated in honey bees (Department for Environment, Food and Rural Affairs [Defra] 2016a, 2016b).

The purpose of the colony feeding study was to evaluate in a field setting (albeit a worst-case field setting where thiamethoxam-treated sucrose was fed directly into the hives, and there was very limited alternative forage) the potential effect of thiamethoxam exposure on honey bee brood and other colony-level parameters in free-foraging honey bee colonies. Analyses of the data indicate effects on colony endpoints at the 100 μg/L level following consumption of the treated sucrose solution; reductions in consumption of the 100 μg/L treated sucrose reflected the reduced consumption by adult bees in the laboratory. The effects of exposure at the colony level were consistent across adult bees, all brood stages, and the bee bread stores and occurred from the dosing period to the start of overwintering.

Significant effects in various endpoints were also observed at the 50 and 37.5 μg/L treatment levels; however, these effects were transient and did not result in significant reduction in hive strength (number of adults or total brood) over the course of the study. Further work is required to determine the effects of thiamethoxam on overwintered colonies because most of the colonies in the thiamethoxam treatments and control did not survive as a result of insufficient feeding in late summer/early fall leading to inadequate colony size (i.e., small winter cluster size) and food stores. These data are in contrast with those for another study where thiamethoxam (5.31 ± 0.75 μg/kg) and clothianidin (2.05 ± 1.18 μg/kg) were fed via pollen patties (the dose was prepared in sucrose mixed into the pollen) over 2 brood cycles (6 wk; Sandrock et al. 2014), and colonies exhibited decreased performance in the short term, resulting in declining numbers of adult bees, brood, honey production, and pollen collection; colonies recovered in the medium term and overwintered successfully. One significant difference between the designs of the 2 thiamethoxam studies that may explain the contradictory findings was that pollen traps were placed on the pollen-fed colonies throughout the feeding period (Sandrock et al. 2014), severely restricting access to any pollen other than that fed in irradiated patties; thus, effects may be compounded by pollen quality (Renzi et al. 2016). This reported effect at low doses supplied via pollen patties is also contrary to the findings for another neonicotinoid (imidacloprid) which established the NOAEC for effects on colonies as 100 μg/kg pollen (Dively et al. 2015). The present data for sucrose-fed colonies support the lack of effects identified in previous colony-level field studies with thiamethoxam (Pilling et al. 2013; Henry et al. 2015) as well as imidacloprid fed in sucrose (Faucon et al. 2005) and pollen patties (Dively et al. 2015) and following exposure to clothianidin-treated spring oilseed rape (Rundlof et al. 2015). However, unlike these studies demonstrating no effects, these data also provide a threshold level of exposure likely to result in adverse effects on the colony with minimal alternative forage.

There has been interest in the use of sublethal effects from laboratory studies to predict field-level effects, and the present feeding study offers such an opportunity to relate residues in pollen and nectar following treatment of crops to short-medium-term colony-level effects; longer-term effects cannot be assessed with the present study based on the high levels of mortality across control and treatments over winter. Many of the individual sublethal effects observed in the laboratory, for example, proboscis extension reflex, have been difficult to link directly to impact at the colony level (Thompson 2003). One of the more recent parameters identified has been the development of the hypopharyngeal gland as a potential indicator of the ability of adult nurse bees to feed brood, but it is unclear what scale of effect in the laboratory is adverse in relation to colonies under field conditions. The acini in the hypopharyngeal glands in 12-d-old honey bees consuming 10 and 40 μg thiamethoxam/L were 7.5 to 13.2% smaller than those of control bees (Renzi et al. 2016). If the ability of the nurse bees to produce brood food was impacted, then effects may be expected on brood production at similar concentrations. However, in the present colony feeding study, there were no statistically significant effects on brood observed after 6 wk of feeding with up to 37.5 μg thiamethoxam/L, suggesting that the decrease observed in the hypopharyngeal gland in the laboratory study cannot be directly linked to an impact at the colony level. Interestingly, the concentration of thiamethoxam in sucrose that resulted in adverse effects in the present colony feeding study (>50 μg/L) is comparable to that previously reported when fed directly to individual foragers with consequent impacts on homing behavior in the field (64 μg/L; Henry et al. 2012), which suggests that effects are observed only following consumption of higher concentrations of thiamethoxam.

In comparing residues of thiamethoxam in pollen and nectar from treated crops to those used within the feeding study, we propose an approach of considering the worst-case exposure profile in terms of total exposure to residues of the different castes of honey bees within the hive (Sponsler and Johnson 2017) in relation to the proportion of pollen and nectar consumed (US Environmental Protection Agency 2014). This approach has the same basis as other areas of toxicology and ecotoxicology because, the source of the residues being disregarded, it is the total exposure concentration that is relevant. If higher residues are detected in pollen than nectar, then the exposure profile of in-hive bees is the worst case (in-hive bees estimated to consume 90% nectar:10% pollen [Rortais et al. 2005]); if higher residues are detected in nectar than pollen, then the forager is the worst case (nectar foragers consume 100% nectar [Rortais et al. 2005]). Thus, for oilseed rape grown from thiamethoxam-treated seed, the median residues were 2 μg/kg nectar and 3 μg/kg pollen (Pilling et al. 2013), and the worst-case exposure profile would be for in-hive bees, which would have consumed 2.1 μg thiamethoxam/kg.

The present feeding study was conducted under worst-case conditions with an extended feeding period representing access to a single long-flowering crop with very limited alternative forage, while ensuring that the colonies still needed to forage for pollen required for brood production. Consistent exposure was demonstrated by the high residues in both bee bread and nectar stores within the colonies at the end of the 6-wk feeding period.

Apart from the lack of sufficient feeding prior to the overwintering period, the colonies can be considered representative of normal well-managed colonies as they contained parasites (*Nosema* and low levels of *Varroa*) and were also exposed to other pesticides within the environment. Typical colony maintenance feeding with sucrose solution (2:1, sucrose:water) starts in August in the piedmont of North Carolina, where the present study was conducted. The delay in maintenance feeding (after CCA 8; 27 October) was the result of concerns by scientists from the regulatory agencies, for which the present study was being conducted, that the test item could be diluted with the addition of uncontaminated sucrose solution provided as a food source or that stores of the nectar containing the test item might not be consumed if the bees preferred the uncontaminated sucrose solution. However, if an objective of a study is assessing potential effects of a test item on the overwintering success of a colony, the results of the overwintering aspect of the present study demonstrate that appropriate colony maintenance according to local standards needs to be prioritized over any potential impact of the maintenance on the exposure of the test item to the bees.

Despite the consistent long-term exposure, the only adverse colony effects reported occurred at 100 μg thiamethoxam/L (79 μg thiamethoxam/kg); all other significant effects determined at other treatment levels were transient and did not impact the colony strength compared with controls. This colony NOAEC of 50 μg thiamethoxam/L (43 μg thiamethoxam/kg) provides a basis by which to evaluate the potential risk of thiamethoxam residues detected in pollen, nectar, or water following treatment of bee-attractive crops. It also provides additional support for the lack of effects reported in field studies following exposure of colonies to levels of thiamethoxam in pollen and nectar of seed-treated crops (Pilling et al. 2013) that are an order of magnitude lower than the no-effect level observed in the present study.

Supplemental Data—The Supplemental Data are available on the Wiley Online Library at DOI: 10.1002/etc.4018.

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Data availability—Data are available upon request for noncommercial purposes only from the corresponding author (jay.overmyer@syngenta.com).

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