Bulletin of Insectology **65** *(1): 99-106, 2012*
ISSN 1721-8861

In situ replication of honey bee colony collapse disorder

Chensheng LU[1], Kenneth M. WARCHOL[2], Richard A. CALLAHAN[3]
[1]*Department of Environmental Health, Harvard School of Public Health, Landmark Center West, Boston, MA, USA*
[2]*Worcester County Beekeepers Association, Northbridge, MA, USA*
[3]*Worcester County Beekeepers Association, Holden, MA, USA*

Abstract

The concern of persistent loss of honey bee (*Apis mellifera* L.) colonies worldwide since 2006, a phenomenon referred to as colony collapse disorder (CCD), has led us to investigate the role of imidacloprid, one of the neonicotinoid insecticides, in the emergence of CCD. CCD is commonly characterized by the sudden disappearance of honey bees (specifically worker bees) from hives containing adequate food and various stages of brood in abandoned colonies that are not occupied by honey bees from other colonies. This *in situ* study was designed to replicate CCD based on a plausible mechanistic hypothesis in which the occurrence of CCD since 2006 was resulted from the presence of imidacloprid, one of the neonicotinoid insecticides, in high-fructose corn syrup (HFCS), fed to honey bees as an alternative to sucrose-based food. We used a replicated split-plot design consisting of 4 independent apiary sites. Each apiary consisted of 4 different imidacloprid-treated hives and a control hive. The dosages used in this study were determined to reflect imidacloprid residue levels reported in the environment previously. All hives had no diseases of symptoms of parasitism during the 13-week dosing regime, and were alive 12 weeks afterward. However, 15 of 16 imidacloprid-treated hives (94%) were dead across 4 apiaries 23 weeks post imidacloprid dosing. Dead hives were remarkably empty except for stores of food and some pollen left, a resemblance of CCD. Data from this *in situ* study provide convincing evidence that exposure to sub-lethal levels of imidacloprid in HFCS causes honey bees to exhibit symptoms consistent to CCD 23 weeks post imidacloprid dosing. The survival of the control hives managed alongside with the pesticide-treated hives unequivocally augments this conclusion. The observed delayed mortality in honey bees caused by imidacloprid in HFCS is a novel and plausible mechanism for CCD, and should be validated in future studies.

Key words: colony collapse disorder, imidacloprid, *Apis mellifera*, neonicotinoid insecticides, high-fructose corn syrup.

Introduction

The abrupt emergence of colony collapse disorder (CCD) in the United States during 2006-2007 (vanEngelsdorp *et al*., 2007; 2008), and other countries later (Bacandritsos *et al*., 2010) has raised the concern of losing this important perennial pollinator globally. The persistence of CCD worldwide was highlighted in a recent United Nations report (UN News Center, 2011), which calls for changes in honey bee colony management in order to save this important insect. CCD is commonly characterized by the sudden disappearance of honey bees (specifically worker bees) from hives containing adequate food (e.g. honey, nectar, and pollen) and various stages of brood in abandoned colonies that are not robbed by honey bees from other colonies, as described in a recent review article (Spivak *et al*., 2011). Although some losses of honey bees from healthy and well managed hives during the winter months have always been part of apiculture (for instance, in the New England area, winter losses of honey bee hives are typically 15-30%), never in the history of the beekeeping industry has the loss of honey bee hives occurred in such magnitude and over such a widely distributed geographic area.

The honey bee (*Apis mellifera* L.) is an insect that has evolved the ability to survive winters by forming a cluster of thousands of bees that cooperatively generate heat with their thoracic muscles. Temperatures within a cluster can and often exceed 32 °C when the outside temperature is well below freezing. Honey bees obtain the needed energy from sugar stored as honey or supplemental sugar-based alternatives supplied by beekeepers. Worker caste bees that emerge in the summer typically live about 40 days, whereas those emerging in September through November will live up to 200 days and consume significant stores of food, mostly honey, throughout the winter months (Robinson *et al*., 2005; Patel *et al*., 2007). In the fall, honey bees migrate to the bottom of their hive and as the temperature continues to drop, bees cluster under their honey stores. Heat lost from the cluster rises to warm the honey immediately above it. As the winter season progresses, the cluster moves upward consuming the warmed honey immediately above, however, bees are limited in their ability to consume cold honey to the side of the cluster. Winter losses of honey bee hives usually occur because honey bees run out of or cannot access food, or the cluster becomes too small to generate sufficient heat.

A long list of biological, chemical, and environmental stressors has been linked to CCD, including *Varroa* mites (de Miranda *et al*., 2010), Israel acute paralysis virus (Cox-Foster, 2007; Blanchard *et al*., 2008), *Nosema ceranae* (Higes *et al*., 2008), and exposure to systemic neonicotinoid insecticides, e.g. imidacloprid (Girolami *et al*., 2009; Maini *et al*., 2010). The practices of migratory commercial beekeeping, which often involve moving hives long distances to pollination sites, and malnutrition associated with monocultural food sources, have also been blamed for causing CCD (Spivak *et al*., 2011). Although a recent report concludes that biotic factors (e.g., pests and pathogens) are most likely responsible for the extensive loss of honey bee colonies, such a conclusion remains debatable considering these stressors

have been associated with beekeeping for decades and are as common among sedentary as migratory colonies (Neumann and Carreck, 2010). None of these potential culprits, either alone or in combination, has been demonstrated to trigger the symptoms of CCD. Therefore, the status of CCD research is best summarized in a recent article as: "Most reports express opinions but little hard science" (Ratnieks and Carreck, 2010).

This *in situ* study was designed to replicate CCD based on a plausible mechanistic hypothesis that has not yet been discussed widely. We hypothesized that the first occurrence of CCD in 2006/2007 resulted from the presence of imidacloprid (1-((6chloro-3-pyridinyl) methyl)-N-nitro-2-imidazolidinimine, CAS# 138261-41-3), in high-fructose corn syrup (HFCS), fed to honey bees as an alternative to sucrose-based food. There are three facts to support this hypothesis. First, since most of the suspected but creditable causes for CCD were not new to apiculture, there must have been an additional new stressor introduced to honey bee hives contemporaneous with the first occurrence of CCD during the winter months of 2006 and early 2007. Second, while commercial beekeepers appear to be affected by CCD at a disproportional rate, their beekeeping practices have been relatively unchanged during these years except for the replacement of honey or sucrose with HFCS as the supplemental sugar source for economic and convenient reasons. This is because many of the commercial beekeepers leave very little honey in their hives to sustain honey bees through the winter months, and therefore require the least expensive alternative for honey. Although the replacement of honey/sucrose-based feeds with HFCS among commercial beekeepers took place much earlier than 2006/2007, it was the timing of the introduction of neonicotinoid insecticides to the corn-seed treatment program first occurring in 2004/2005 that coincides with CCD emergence (Bonmatin *et al*., 2005; Benbrook, 2008). Lastly, several earlier reports have shown that corn and sunflower plants grown from genetically engineered seeds treated with imidacloprid, one of the neonicotinoid insecticides, produce pollen with average levels of 2.1 and 3 μg/kg of imidacloprid, respectively (Suchail *et al*., 2001, Rortais *et al*., 2005). Furthermore, a recent paper published during the course of this *in situ* study showed elevated imidacloprid residue levels of 47 mg/L in seedling corn guttation drops germinated from seeds treated with 3 different neonicotinoid insecticides-treated (including imidacloprid) corn plants that are high enough to kill honey bees instantaneously (Girolami *et al*., 2009). These study results lend credence to our hypothesis that the systemic property of imidacloprid is capable of being translocated from treated seeds to the whole plant, including corn kernels and therefore likely into HFCS. The widespread planting of genetically engineered corn seeds treated with elevated levels of neonicotinoid insecticides, such as imidacloprid since 2004 (Van Duyn, 2004), and their acute toxicity to honey bees led us to hypothesize a link between CCD and feeding of HFCS containing neonicotinoid insecticides. It should be noted here that the residue levels of imidacloprid, or other neonicotinoid insecticides, have not been routinely monitored in HFCS.

Materials and methods

We used brand new hive materials, as well as new honey bee packages to minimize any possibility of unknown pesticide residues or diseases present in existing honey bee colonies. We used a replicated split-plot design consisting of 4 sites with 5 honey bee hives on each site. Study sites were located at least 12 km away from each other; therefore, each study site is considered an independent apiary. Each apiary consisted of 4 different imidacloprid-treated hives and a control hive, which was managed identically to the treated hives except no imidacloprid was added to its HFCS. The dosing regime was initiated after each of the 20 hives consisted of at least 15 frames of bees and all 20 frames of comb were drawn. The dosages used in this study were determined to reflect imidacloprid residue levels reported previously (Suchail *et al*., 2001; Bonmatin *et al*., 2005; Rortais *et al*., 2005; Girolami *et al*., 2009). Imidacloprid was initially fed to honey bees at 0.1, 1.1, 5.3, and 10.5 μg/kg in HFCS per week for 4 weeks starting on July 1st 2010, followed by 20, 40, 200, and 400 μg/kg per week for additional 9 weeks, which ended on September 30th 2010. The field investigators were blind to the dosing regime in order to minimize bias and subjective assessment. This *in situ* study involving the use of honey bees was reviewed and waived by Harvard School of Medicine Animal Care Committee.

Preparation of honey bee hives

Twenty, new 10-frame Langstroth pine hives were made (Humble Abodes Inc., Windsor ME), assembled (Autumn Morning Farm, Barre MA), and painted externally with white latex paint. Each hive consisted of two deep hive bodies, a telescoping, metal clad outer and a vented inner cover, a bottom board and a hive stand. A third deep hive body was provided to house syrup feeding bottles. Five hives were setup in each of four apiaries about 12 kilometers apart in southern Worcester County located in Central Massachusetts, USA. This separation was sufficient to isolate one apiary from the other. At each apiary the five hives were set upon two parallel sixteen foot 4 × 4 leveled timbers about 40 cm off the ground with a slight forward pitch according to standard practice. Hives faced south to southeast and had a windbreak to their rear, either a structure or evergreen trees. Wax foundation (Walter T. Kelley Bee Co., Clarkson, KY) was installed on 21.59 × 42.55 cm pine frames and placed in the hive bodies.

Twenty packages (each weighing approximately 1.4 kg) of Italian honey bees (Rossman Apiaries Inc., Moultrie, Georgia) were installed in the bottom hive body on March 28th, 2010. All hives were fed with HFCS from plastic frame feeder (Mother Loader Products, Sonora CA). Hives were monitored weekly, and managed using standard beekeeping techniques. These included balancing hives within each apiary by moving brood between hives during the setup period and preventing so called "honey-bound" conditions. During this setup period, 6 nonperforming queens (2 for apiary #1 and #3 and 1 for apiary #2 and #4) were replaced with queens obtained from Rossman Apiaries. By May 21st, 2010 all twenty

frames in each of 20 hives were drawn out into comb and contained at least 14 frames of capped brood. No further movement of frames between hives was allowed after May 21st, 2010.

Imidacloprid administration via HFCS

Imidacloprid (Catalog No. PS-2086, Chem Service, Inc. West Chester, PA) was dissolved in methanol to form a stock solution, and then diluted in 4-ml glass vials to four pre-determined dosages, plus a control with no imidacloprid added, in de-ionized water before adding to HFCS on site (table 1). Glass vials were labeled 1-5, the corresponding to hive ID numbers at each of the 4 apiaries. The imidacloprid dosing regime was blind to field investigators. On each dosing day, each vial was mixed into one glass jar containing approximately 2.6 kg of HFCS and fitted with metal screw caps (AB Container, Enfield CT). The glass jars were set upon the inner covers of the hives. Honey bee obtained HFCS through holes drilled in the caps. The imidacloprid dosages delivered to the hives were confirmed in the quality assurance/quality control program (table 2).

Apiaries were numbered 1-4 and hives were numbered 1-5 such that hive ID#1-1 was referred as the far-left hand hive at apiary 1 and hive ID#4-5 was referred as the far-right hand hive at apiary 4. Treatments were repeated weekly from July 1st - September 30th, 2010. Unused syrup was measured and discarded and exposure calculations adjusted accordingly, although the incomplete consumption of HFCS rarely occurred. After September 30th, 2010 all hives were fed with blank HFCS to ensure that all hives had at least fifteen frames of stored food for the winter.

Monitoring brood production

A number of factors could influence the production of brood in a healthy hive including availability of nectar and pollen, availability of open cells for egg laying, numbers of nurse bees, and overall vitality and quality of the queen. From July 7th to September 30th 2010, the brood production of all hives was assessed on a bi-weekly basis. All hives at two of the four apiaries were assessed weekly using a modification of the brood assessment method (Emsen, 2005). The twenty frames in each hive were scored cumulatively for the area covered by "sealed brood". Sealed brood is the pupal stage of honey bee development and for the worker caste extends for fourteen days. This bi-weekly assessment therefore provides an objective measurement of each colony's brood rearing. Brood was estimated by dividing the face of each side of frame into 32 squares (each square containing approximately 100 cells). All 20 frames in each hive were scored by visually estimating the number of squares of capped brood per frame face. Two hives from each treatment group were scored per week. The alternate two hives were assessed the following week. During this scoring process notes were also made of the number of frames of adult bees observed. No other procedures were implemented during the imidacloprid dosing months.

Treatment for parasites and winter monitoring

Two Apistan strips (Mann Lake Ltd., Hackensack, MN) were placed next to brood to control *Varroa* mite on October 5th, 2010 in all hives and then removed on November 20th, 2010. During the same period, all hives were fed 7.6 litres of blank HFCS containing 9.1 g

Table 1. The weekly administration of imidacloprid (µg/kg) in high-fructose corn syrup (HFCS) and the total imidacloprid dose (µg) delivered to each honey bee hives [1].

Imidacloprid dosages	Hive ID#				
	1	2	3	4	5
Initial dosage (µg/kg of HFCS) per week for 4 weeks	10.5	5.3	1.1	0.1	Control
Amount of imidacloprid delivered to each hive per week (µg) [2]	26	13	2.6	0.26	0 [3]
Total amount of imidacloprid delivered to each hive during the first 4 weeks (µg)	104	52	10.4	1.04	0
Follow-up dosage (µg/kg of HFCS) per week for 9 weeks	400	200	40	20	Control
Amount of imidacloprid delivered to each hive per week (µg) [2]	1,038	519	103.8	51.9	0 [3]
Total amount of imidacloprid delivered to each hive during the follow-up 9 weeks (µg)	9,342	4,671	934.2	467.1	0
Total amount of imidacloprid delivered to each hive during the 13 weeks (µg) [4]	9,446	4,723	944.6	468.1	0

[1] The dosages corresponding to individual hive ID# were applied to 4 apiaries.
[2] Aliquot (3mL) of imidacloprid dissolved in methanol was added to 1.9 litres of HFCS which weighs 2.59 kg. This is the weekly dosage that is delivered to the corresponding hive.
[3] Only aliquot (3mL) of methanol was added to HFCS.
[4] The sum of imidacloprid (µg) delivered to each hive for the entire 13 weeks.

Table 2. Recoveries of imidacloprid in high-fructose corn syrup (HFCS) prepared in the quality assurance/quality control program[1].

Sample type	Imidacloprid (μg/kg)	Sample size	Recovery (%)[2]
Quality control[3]	2 - 25	12	114 (11.8)
Quality assurance[4]	0.5 - 200	9	97 (13.5)
Blank HFCS[5]	n.a. [6]	6	n.a.

[1] Imidacloprid in HFCS analyzed using method published by Zhang *et al*. (2011).
[2] Standard deviation for the respective recovery in the parenthesis.
[3] Fortifying HFCS used in this study with known amount of imidacloprid in the laboratory.
[4] HFCS samples with various imidacloprid dosages collected from the field.
[5] The original HFCS samples used in this study.
[6] Contained imidacloprid levels below the limit of detection at 0.1 μg/kg.

Fumagillin B (Medivet Pharmaceuticals Ltd., High River, Alberta Canada) to control *Nosema apis* and *Nosema ceranae*, two common intestinal parasites. Entrance reducers were also installed.

The survival of all hives was monitored weekly beginning in December 3rd, 2010. Starting December 22nd 2010, hives stores were supplemented with crystallized HFCS mixed into a paste with granular sucrose. The food was placed on waxed paper on top of the frames inside the inner covers. Notes were taken on the general appearance and size of the clusters observed. As soon as a hive was identified as a dead hive, food was removed and the entry to the hive was sealed with duck tape to prevent early spring robbing by other honey bees.

Results

The timeline of this experiment, including the dates of observed events, is shown in table 3. We assessed brood rearing by estimating the number of sealed brood in all 20 frames of each hive on a bi-weekly basis from July to the end of September 2010. We found that the initial brood rearing corresponded to imidacloprid doses two weeks after the initial imidacloprid dosing, however, it is inversely related to imidacloprid dosages at the end of dosing regime (figure 1). The number of sealed brood for both treated and control hives decreased significantly from July to September (GLM, $p < 0.001$), however this decrease is independent of different imidacloprid doses applied to the hives. It should be noted that the steady decreasing trend of sealed brood during the

Table 3. The progression of the *in situ* study and the dates of dead honey bee hive observation.

Date	Event
Jan-Feb, 2010	Assembling 20 new 10-frame Langstroth pine honey bee hives.
March, 2010	Study site selection and apiary setup.
March 28th, 2010	Introducing honey bees (bee shaking) to 20 new hives in 4 apiaries.
May 21st, 2010	All 20 hives contained at least 15 frames of capped brood.
July 1st - 29th, 2010	Initial low imidacloprid dosing for 4 consecutive weeks.
July 29th - Sept 30th, 2010	Follow-up high imidacloprid dosing for 9 consecutive weeks.
July-Sept, 2010	Monitoring strength of honey bee hives biweekly.
Oct 5th - Nov 20th, 2010	Parasite treatment (Apistan strips and Fumagillin B) on all hives.
Dec 3rd, 2010 - present[1]	Winter hive strength monitoring.
Dec 22nd, 2010 - present[1]	Feeding hives with crystallized HFCS mixed with granular sucrose.
Dec 22nd, 2010	Last monitoring date without the observation of dead hives.
Dec 31st, 2010	The 1st and 2nd hives treated with 400 μg/kg imidacloprid dose dead.
Jan 7th, 2011	The 1st hive treated with 40 μg/kg imidacloprid dose dead.
Jan 14th, 2011	The 1st hive treated with 200 μg/kg imidacloprid dose dead.
Jan 19th, 2011	The 2nd hive treated with 200 μg/kg imidacloprid dose dead.
Feb 4th, 2011	The 3rd and 4th hives treated with 400 μg/kg imidacloprid dose dead. The 2nd hive treated with 40 μg/kg imidacloprid dose dead.
Feb 24th, 2011	The 3rd, 3rd and 4th, and 1st and 2nd hives treated with 200, 40, and 20 μg/kg imidacloprid dose, respectively dead. The 1st control hive dead.
March 10th, 2011	The 4th and 3rd hive treated with 200 and 20 μg/kg imidacloprid dose, respectively, dead. The 4th hive treated with 40 μg/kg imidacloprid and 3 control hives remain alive.

[1] On-going activities as of March 21st, 2011.

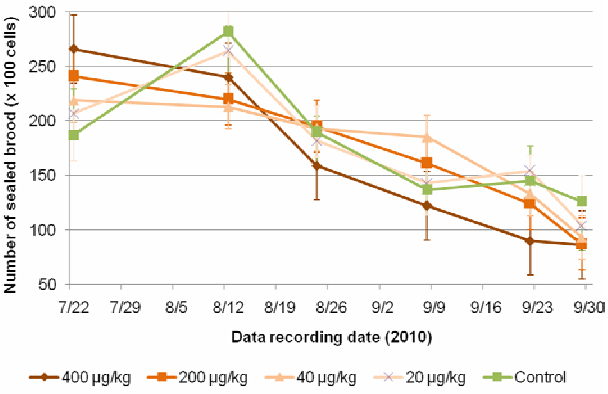

Figure 1. The average estimated numbers of sealed brood of four honey bee hives for each of four imidacloprid dosages and the controls. Data were recorded every two weeks from July to September 2010.

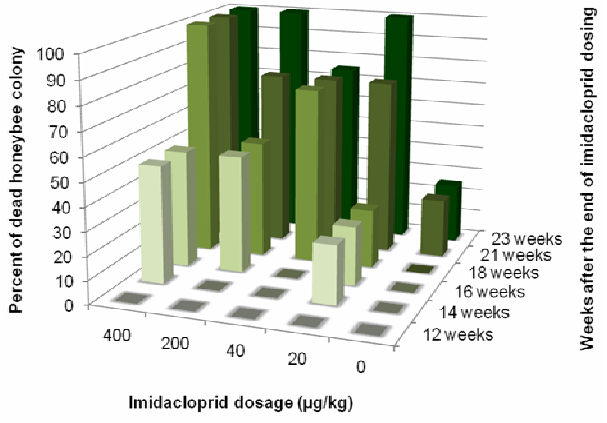

Figure 2. The progression of honey bee hive mortality associated with imidacloprid dosages and the control 23 weeks post imidacloprid dosing. Each imidacloprid-treated group and the controls included four hives placed in four different apiaries.

summer months as observed in this study is vastly different from that normally seen in honey bee hives residing in the central Massachusetts area. Under normal growing conditions, brood rearing in well-managed hives often begins in mid-January and builds exponentially until mid-June. Typically, brood rearing levels off until mid-July, and then takes a slight dip due to the nectar dirth that usually continues until early August at which point there is a slight brief resurgence in brood rearing before leveling off in late August. Brood rearing takes a quick last surge in September until mid-October at which point there is a quick decline with brood rearing ending in November.

All twenty hives were alive when they were assessed on December 22nd 2010, 12 weeks post imidacloprid dosing (PID), although at this time the strength of hives treated with the highest imidacloprid dose appeared to be weakening as observed by smaller clusters and frozen dead honey bees scattering (on snow) in front of the hives. The first observation of two dead hives was recorded 13 weeks PID (table 3). Additional imidacloprid-treated hives began to show signs of weakness throughout January 2011. Significant loss of hives did not occur until 18 weeks PID in which during the following 5-week period, additional 8 hives treated with various imidacloprid doses died. All control hives remained alive 18 weeks PID. Three additional imidacloprid-treated hives and the first control hive died 21 weeks PID. Twenty-three weeks PID, only 1 imidacloprid-treated hive remained alive, whereas 3 of the four control hives were alive. Figure 2 shows the progression of hive mortality associated with different imidacloprid dosages 23 weeks PID.

Discussion

The magnitude and the pattern of honey bee hive loss during the winter months in this study resemble the reported symptoms of CCD. The loss of 15 of 16 imidacloprid-treated hives (94%) across 4 apiaries occurred over a period of 10 weeks following the first hive death. Dead hives were remarkably empty except for stores of food and some pollen left on the frames (figure 3). The dead hives, particularly for those treated with higher dosages of imidacloprid, was preceded by the observation of dead bees scattered on snow in front of the hives, with diminished small clusters remaining the week before death. Snow usually fell between weekly hive examinations making the observation of scattered dead honey bees in front of individual hives noticeable. Although this observation is not quite reminiscent of the reported CCD symptoms, it is important to consider that if these hives were located in a warmer climate region, such as in Florida USA where migratory hives overwinter, bees exiting the hives would have dispersed some distance from the hives and therefore would not be observed in front of the hives.

The replicated controlled design of this *in situ* study in the apiarian setting, and the survival of honey bees in 3 of 4 control hives (figure 4), eliminate the possibility that hive deaths were caused by common suggested risk factors, such as long-distance transportation of hives, malnutrition, or the reported toxic effect of hydroxymethylfurfural, a heat-formed contaminant during the distillation process of making HFCS, to honey bees (LeBlanc *et al.*, 2009). We used the same HFCS in both the imidacloprid-treated and control hives. The loss of imidacloprid-treated hives in this study is also highly unlikely due to pathogen infection since the presence of neither *Nosema* nor a large number of *Varroa* mites was observed in hives during the summer and fall seasons. In addition, all hives were treated with Apistan strips and Fumagillin B, two effective treatments for parasite prevention, prior to the winter season. Since all hives were considered healthy as they went into fall season, those pathogens posed very little threat to the health of honey bee hives. The only dead control hive exhibited symptoms of dysentery in which dead honey bees were found both inside and outside of the hive, which is not seen in the other 19 hives.

Data from this *in situ* study provide convincing evidence that exposure to sub-lethal levels of imidacloprid

Figure 3. Dead hive (ID# 4-4) treated with 20 µg/kg of imidacloprid which shows the abundance of stored honey and some pollen, but no sealed brood or honey bees. Photo was taken on February 24th, 2011.

Figure 4. Control hive (ID# 2-5), which shows a cluster of honey bees, some stored honey and uncapped larvae, but no sealed brood. Photo was taken on March 4th, 2011.

causes honey bees to exhibit symptoms consistent to CCD months after imidacloprid exposure. Should stressor factors other than feeding honey bees with HFCS containing imidacloprid cause CCD, the loss of honey bees would not occur disproportionally on those imidacloprid-treated hives. The survival of the control hives unequivocally augments this conclusion. The study hypothesis is further supported by the mortality data presented in figure 2, which clearly demonstrates a dose-response relationship, in which the highest imidacloprid dose exterminates hives more quickly than the subsequent doses in all 4 apiaries. Although imidacloprid, and other neonicotinoid insecticides have been suggested as a possible contributing factor to CCD because of its toxicity in impairing foraging ability or triggering other neuro-behavioral problems (e.g. failure to return to the hive) in honey bees at sub-lethal doses (Suchail *et al*., 2001; Rortais *et al*., 2005; Thompson and Maus, 2007; Yang *et al*., 2008; Mullin *et al*., 2010), its attribution to CCD in the apiary setting has never been documented. The results from this study underscore the paucity of research concerning the sub-lethal effects of pesticides on CCD, particularly of neonicotinoids throughout the yearly life cycle of entire honey bee colonies under natural conditions (Maini *et al*., 2010; Spivak *et al*., 2011).

One apparent deficiency, in addition to the small number of honey bee hives used in this study, is that we were not able to obtain HFCS manufactured in 2005/2006 for use in this experiment. Instead, we used food-grade HFCS fortified with different levels of imidacloprid, mimicking the levels that are assumed to have been present in the older HFCS. The range of dosages used in this study from 20 to 400 µg/kg were not only environmentally relevant to those reported imidacloprid levels by studies that are cited previous, but also lie within legally allowable levels, set by the US Environmental Protection Agency (EPA) as the tolerance of 0.05 ppm (50 µg/kg) for corn (US CFR, 2010). Since there is no tolerance level for imidacloprid in HFCS, we applied a 10-fold concentrating factor, or 0.5 ppm (500 µg/kg) of imidacloprid in HFCS, by taking into account the uptake by corn plants from seeds that are treated with imidacloprid. The 10-fold concentrating factor is very conservative compared to the reported average level of 47 mg/L of imidacloprid measured in guttation drops collected from corn seedlings germinated from commercial seeds obtained in 2008 coated with 0.5 mg/seed of imidacloprid (Girolami *et al*., 2009). Considering that honey bees were diluting the concentrations of imidacloprid fed to the hives with natural nectars foraged during the HFCS feeding months (July to September), honey bees may have exposed to imidacloprid at the dosage lower than 20 µg/kg in which is sufficient to render mortality in honey bees. Therefore, we are confident that the imidacloprid dosages applied in this study would be comparable, if not lower to those encountered by honey bees inside and outside of their hives. Nevertheless, the finding of the loss of honey bee hives at the levels as low as 20 µg/kg of imidacloprid in HFCS raises the question of whether there is a no-observed-adverse-effect-level of imidacloprid (and most likely of other neonicotinoids as well) for honey bees.

There are several questions that remain unanswered as a result of this study. First, the systematic loss of sealed brood in the imidacloprid-treated and control hives may indicate a common stress factor that was present across all 4 apiaries. Although brood rearing is known to be affected by various field conditions, such as available cells for egg laying, availability of nectar and pollen, temperature, and the age and quality of honey bee queen, the continuous decrease of brood rearing over the summer month raises the question of whether feeding honey bees with HFCS would compromise the quality of brood rearing in the hives. This concern is relevant to apiculture since CCD is often linked to feeding honey bees with a monoculture diet either from pollinating a single crop (e.g., almonds) or *via* a single sugar-based food source, like HFCS.

Second, while it is apparent that honey bees died during the winter months did not directly consume HFCS containing imidacloprid when it was fed during the summer months, the delayed mortality in honey bees observed in late winter months remains puzzling. One plausible explanation is that these adult honey bees, which emerged in late summer/early fall, were exposed

to imidacloprid during their larval stage, and the toxicity of imidacloprid at the sub-lethal levels was later manifested in the adult honey bees. Results from a recent *in vitro* study (Medrzycki *et al*., 2010) alluded to a mechanism that may relate to CCD caused by imidacloprid in HFCS. Medrzycki *et al.* demonstrated a link between the quality of the brood rearing environment and both the reduction in longevity and the susceptibility to an insecticide in adult honey bees emerging from their larvae. They reported that by lowering the brood rearing temperature 2 °C from the optimal 35 °C, it strongly affected adult honey bees' mortality and their susceptibility to dimethoate, an organophosphate insecticide. Since it is well known that the physiology of adult honey bees can be affected by the health of their larvae and/or pupae, it implies that the onset of CCD as a result of delayed mortality in adult honey bees may start in the larval stage. The feeding of HFCS containing imidacloprid throughout honey bees' life cycle may initiate CCD by compromising larval development throughout the summer and early fall months as observed in this *in situ* study (figure 1). The presence of imidacloprid in HFCS subsequently renders additional susceptibility, in the form of shorter longevity, to adult honey bees that emerged in early fall. The loss of honey bees due to shorter longevity during the winter months would have no doubt affected the size of the cluster, leading to the collapse of imidacloprid-treated honey bee colonies. The delayed mortality phenomenon would therefore be seen in imidacloprid-treated hives, but not in the control hives. If imidacloprid exposure is truly the sole cause of CCD, it might also explain the scenario in which CCD occurred in honey bee hives not fed with HFCS. Considering the sensitivity of honey bees to imidacloprid as demonstrated in this study and the widespread uses of imidacloprid and other neonicotinoid insecticides, pollen, nectar, and guttation drops produced from those plants would have contained sufficient amounts of neonicotinoid insecticide residues to induce CCD (Benbrook, 2008).

From the ecological and apicultural perspectives, the results from this study show a profound and devastating effect of low levels of imidacloprid in HFCS on honey bee colonies. The initial investigations of the causes of CCD focusing on direct exposures *via* foliar or soil application, ingestion of pollen/nectar, or cross-contamination in hives, failed to detect the link of the sub-lethal toxicity of imidacloprid in sugar-based alternative feeds, such as HFCS. By incorporating the findings from this *in situ* study and other reports, we have validated the study hypothesis in which the initial emergence of CCD in 2006/2007 coincided with the introduction of genetically engineered corn seeds treated with imidacloprid and other neonicotinoid insecticides. It is likely that CCD was caused by feeding honey bees with low levels of imidacloprid in HFCS throughout their lifecycle in which toxicity occurred during the larval/pupal stages and was later manifested in the adult honey bees. The proposed mechanism of delayed mortality should be carefully examined and validated in future studies.

Acknowledgements

This study is supported by a grant funded by Harvard University Center for the Environment. Authors would like to thank K. Desjardin, F. Jacobs, D. Lewcon, and J. Rogers who provided space to establish apiaries, and to acknowledge Dr. Charles Benbrook at The Organic Center for his intellectual inputs to this study. Authors declare no competing financial interests.

References

BACANDRITSOS N., GRANATO A., BUDGE G., PAPANASTASIOU I., ROINIOTI E., CALDON M., FALCARO C., GALLINA A., MUTINELLI F., 2010.- Sudden deaths and colony population decline in Greek honey bee colonies.- *Journal of Invertebrate Pathology*, 105: 335-340.

BENBROOK C., 2008.- Prevention, not profit, should drive pest management.- *Pesticides News*, 82: 12-17.

BLANCHARD P., SCHURR F., CELLE O., COUGOULE N., DRAJNUDEL P., THIÉRY R., FAUCON J-P., RIBIÈRE M., 2008.- First detection of Israeli acute paralysis virus (IAPV) in France, a dicistrovirus affecting honeybees (*Apis mellifera*).- *Journal of Invertebrate Pathology*, 99: 348-350.

BONMATIN J. M., MARCHAND P. A., CHARVET R., MOINEAU I., BENGSCH E. R., COLIN M. E., 2005.- Quantification of imidacloprid uptake in maize crops.- *Journal of Agricultural and Food Chemistry*, 53: 5336-5341.

COX-FOSTER D. L., CONLAN S., HOLMES E. C., PALACIOS G., EVANS J. D., MORAN N. A., QUAN P-L., BRIESE T., HORNIG M., GEISER D. M., MARTINSON V., VANENGELSDORP D., KALKSTEIN A. L., DRYSDALE A., HUI J., ZHAI J., CUI L., HUTCHISON S. K., SIMONS J. F., EGHOLM M., PETTIS J. S., LIPKIN W. I., 2007.- A metagenomic survey of microbes in honey bee colony collapse dirsorder.- *Science*, 318: 283-287.

DE MIRANDA J. R., CORDONI G., BUDGE G., 2010.- The acute bee paralysis virus-Kashmir bee virus-Israeli acute paralysis virus complex.- *Journal of Invertebrate Pathology*, 103: S30-S47.

EMSEN B., 2005.- Semi-automated measuring capped brood areas of bee colonies.- *Journal of Animal and Veterinary Advances*, 5: 1229-1232.

GIROLAMI V., MAZZON M., SQUARTINI A., MORI N., MARZARO M., DI BERNARDO A., GREATTI M., GIORIO G., TAPPARO A., 2009.- Translocation of neonicotinoid insecticides from coated seeds to seedling guttation drops: a novel way of intoxication for bees.- *Journal of Economic Entomology*, 102: 1808-1815.

HIGES M., MARTÍN-HERNÁNDEZ R., BOTÍAS C., BAILÓN E. G., GONZÁLEZ-PORTO A. V., BARRIOS L., DEL NOZAL M. J., BERNAL J. L., JIMÉNEZ J. J., PALENCIA P. G., MEANA A., 2008.- How natural infection by Nosema ceranae causes honeybee colony collapse.- *Environmental Microbiology*, 10: 2659-2669.

LEBLANC B. W., EGGLESTON G., SAMMATARO D., CORNETT C., DUFAULT R., DEEBY T., CYR E. St., 2009.- Formation of hydroxymethylfurfural in domestic high-fructose corn syrup and its toxicity to the honey bee (*Apis mellifera*). *Journal of Agricultural and Food Chemistry*, 57: 7369-7376.

MAINI S., MEDRZYCKI P., PORRINI C., 2010.- The puzzle of honey bee losses: a brief review.- *Bulletin of Insectology*, 63 (1):153-160.

MEDRZYCKI P., SGOLASTRA F., BORTOLOTTI L., BOGO G., TOSI S., PADOVANI E., PORRINI C., SABATINI A. G., 2010.- Influence of brood rearing temperature on honey bee development and susceptibility to poisoning by pesticides.- *Journal of Apicultural Research*, 49 (1): 52-59.

MULLIN C. A., FRAZIER M., FRAZIER J. L., ASHCRAFT S., SIMONDS R., VAN ENGELSDORP D., PETTIS J. S., 2010.- High levels of miticides and agrochemicals in North American apiaries: implications for honey bee health.- *PLoS ONE*, 5 (3): e9754.

NEUMANN P., CARRECK N. L., 2010.- Honey bee colony losses.- *Journal of Apicultural Research*, 49: 1-6.

PATEL A., FONDRK M. K., KAFTANOGLU O., EMORE C., HUNT G., FREDERICK K., AMDAM G. V., 2007.- The making of a queen: TOR pathway is a key player in diphenic caste development.- *PLoS ONE*, 2 (6): e509.

RATNIEKS F. L. W., CARRECK N. L., 2010.- Clarity on honey bee collapse?- *Science*, 327: 152-153.

ROBINSON G. E., GROZINGER C. M., WHITFIELD C. W., 2005.- Sociogenomics: social life in molecular terms.- *Nature Reviews Genetics*, 6: 257-270.

RORTAIS A., ARNOLD G., HALM M. P., TOUFFET-BRIENS F., 2005.- Modes of honeybees exposure to systemic insecticides: estimated amounts of contaminated pollen and nectar consumed by different categories of bees.- *Apidologie*, 36: 71-83.

SPIVAK M., MADER E., VAUGHAN M., EULISS N. H. Jr., 2011.- The plight of the bees.- *Environmental Science and Technology*, 45: 34-38.

SUCHAIL S., GUEZ D., BELZUNCES L. P., 2001.- Discrepancy between acute and chronic toxicity induced by imidacloprid and its meatbolites in *Apis mellifera*.- *Environmental Toxicology and Chemistry*, 20: 2482-2486.

THOMPSON H., MAUS C., 2007.- The relevance of sublethal effects in honey bee testing for pesticide risk assessment.- *Pest Management Science*, 63: 1058-1061.

UN News Center, 2011.- *Humans must change behaviour to save bees, vital for food production*.- [online] URL: http://www.un.org/apps/news/story.asp?NewsID=37731&Cr=unep&Cr1 (accessed May 5, 2011).

US CFR (CODE OF FEDERAL REGULATIONS), 2010.- *Imidacloprid; tolerances for residues 40CFR180.472*, [online] URL: http://edocket.access.gpo.gov/cfr_2010/julqtr/40cfr180.472.htm (accessed May 5, 2010).

VAN DUYN J., 2004.- *Neonicotinoid insecticide seed coatings for protection of planted corn kernels and seedlings*.- North Carolina Cooperative Extension Service [online] URL: http://ces.ncsu.edu/plymouth/pubs/ent/CRGROWERS03.html (accessed May 5, 2011).

VANENGELSDORP D., UNDERWOOD R. M., CARON D., HAYES J. Jr., 2007.- An estimate of managed colony losses in the winter of 2006-2007: A report commission by the apiary inspectors of America.- *American Bee Journal*, 147: 599-603.

VANENGELSDORP D., HAYES J. JR., UNDERWOOD R. M., PETTIS J., 2008.- A survey of honey bee colony losses in the U.S., fall 2007 to spring 2008.- *PLoS ONE*, 3 (12): e4071.

YANG E. C., CHUANG Y. C., CHEN Y. L., CHANG L. H., 2008.- Abnormal foraging behavior induced by sublethal dosage of imidacloprid in the honey bee (Hymenoptera: Apidae).- *Journal of Economic Entomology*, 101: 1743-1748.

ZHANG K., WONG J. W., YANG P., TECH K., DIBENEDETTO A. L., LEE N. S., HAYWARD D. G., MAKOVI C. M., KRYNITSKY A. J., BANERJEE K., JAO L., DASGUPTA S., SMOKER M. S., SIMONDS R., SCHREIBER A., 2011.- Multiresidue pesticide analysis of agricultural commodities using acetonitrile salt-out extraction, dispersive solid-phase sample clean-up, and high-performance liquid chromatography-tandem mass spectrometry.- *Journal of Agricultural and Food Chemistry*, 59: 7636-7646.

Authors' addresses: Chensheng (Alex) LU (corresponding author, cslu@hsph.harvard.edu), Department of Environmental Health, Harvard School of Public Health, 401 Park Drive, Landmark Center West, Boston MA 02215, USA; Kenneth M. WARCHOL, Worcester County Beekeepers Association, Northbridge, MA 01534, USA; Richard A. CALLAHAN, Worcester County Beekeepers Association, Holden, MA 01520, USA.

Received November 1, 2011. Accepted February 24, 2012.