Bulletin of Insectology ***67*** *(1): 125-130, 2014*
ISSN 1721-8861

Sub-lethal exposure to neonicotinoids impaired honey bees winterization before proceeding to colony collapse disorder

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Abstract

Honey bee (*Apis mellifera* L.) colony collapse disorder (CCD) that appeared in 2005/2006 still lingers in many parts of the world. Here we show that sub-lethal exposure of neonicotinoids, imidacloprid or clothianidin, affected the winterization of healthy colonies that subsequently leads to CCD. We found honey bees in both control and neonicotinoid-treated groups progressed almost identically through the summer and fall seasons and observed no acute morbidity or mortality in either group until the end of winter. Bees from six of the twelve neonicotinoid-treated colonies had abandoned their hives, and were eventually dead with symptoms resembling CCD. However, we observed a complete opposite phenomenon in the control colonies in which instead of abandonment, they were re-populated quickly with new emerging bees. Only one of the six control colonies was lost due to *Nosema*-like infection. The observations from this study may help to elucidate the mechanisms by which sub-lethal neonicotinoids exposure caused honey bees to vanish from their hives.

Key words: colony collapse disorder, CCD, honey bee, neonicotinoids, imidacloprid, clothianidin.

Introduction

Since its emergence in 2005/2006, the continuing significant losses of honey bees (*Apis mellifera* L.) colonies resulting from the symptomatic disease of colony collapse disorder (CCD) has demonstrated our inability to identify and eradicate the responsible cause(s) of CCD (BBC News, 2013; The New York Times, 2013; vanEngelsdorp *et al*., 2008). While the prevailing opinions suggest the linkage of CCD to multi-factorial causes including pathogen infestation, beekeeping practices (including malnutrition), and pesticide exposure in general (Cox-Foster *et al*., 2007; Blanchard *et al*., 2008; Higes *et al*., 2008; vanEngelsdorp *et al*., 2009; Alaux *et al*., 2010; de Miranda *et al*., 2010; Williams *et al*., 2010; Di Prisco *et al*., 2011; Vidau *et al*., 2011; USDA, 2013), this notion ignores the differential mortality symptoms; in particular hive abandonment in CCD *vs.* diseased colonies. However, recent scientific findings linking CCD with exposure to neonicotinoids, a group of systemic insecticides, appear to be gaining traction (Maini *et al*., 2010; Pareja *et al*., 2011; Lu *et al*., 2012; Farooqui, 2013; Matsumoto, 2013) and have led to new regulatory control (Erickson, 2012). In this study, we extend our previous study (Lu *et al*., 2012) showing that sub-lethal exposure of imidacloprid and clothianidin affected the winterization of healthy honey bee colonies that subsequently leads to CCD.

Materials and methods

In order to investigate the detrimental effects of sub-lethal neonicotinoid exposure in healthy honey bee colonies, we utilized the split-plot lifecycle study design in which honey bees are fed with pre-determined known amounts of neonicotinoids and allowed to freely forage in the environment. We then assessed their hive growth and strength, as well as their mortality and morbidity, throughout the lifecycle including multiple worker bee generations. The setup and management of eighteen study colonies (using 10-frame Langstroth pine hive) in three apiaries in central Massachusetts was identical to that previously described (Lu *et al*., 2012). At each apiary, we separated six colonies into two groups in which honey bees were fed with either sucrose water or high-fructose corn syrup (HFCS) over the study period. Each sugar group consisted of two neonicotinoid-treated and one control colonies replicated in each of the three apiaries. We purchased sucrose from a local food store and HFCS from a beverage company. Both sugar waters made of sucrose and HFCS were analyzed prior to be used in the experiment and found non-detectable residues of neonicotinoids using a published method (Chen *et al*., 2013). Starting from July 2nd 2012, we administered 258 µg of imidacloprid (1-(6-chloro-3-pyridinyl) methyl)-N-nitro-2-imidazolidinimine, CAS# 138261-41-3) or clothianidin (1-(2-chloro-1,3-thiazole-5-ylmethyl)-3-methyl-2-nitroguanidine, CAS# 210880-92-5) in 1.9 liter (0.5 gallon) of sucrose water and HFCS to the treated colonies each week, respectively, for thirteen consecutive weeks ending on September 17th 2012. Assuming each colony consisted of 50,000 bees at any given day in spring and summer, we administered 0.74 ng/bee/day of either imidacloprid or clothianidin to treated hives for 13 consecutive weeks. This dosage is far below the oral LD50 of 3.4 and 118.7 ng/bee for clothianidin and imidacloprid, respectively (Laurino *et al*., 2013). Control colonies were given neonicotinoid-free sucrose or HFCS throughout the experimental period. Sugar water (both types) was completely consumed by each colony at the end of each week during the 13-week neonicotinoids administration.

From June 29th to September 24th 2012, we assessed

the brood rearing production of all colonies on a bi-weekly basis using a modified brood assessment method as previously described (Lu *et al*., 2012). In brief, the 20-frames in each hive were scored cumulatively for the area covered by "sealed brood" which is the pupal stage of honey bee development. 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. All colonies were treated with Miteaway Quick strips for controlling *Varroa* mite on August 13th 2012, followed by Apistan strips from October 1st to November 15th 2012. The *Varroa* mite counts were assessed twice using the common alcohol wash method on August 13th (pre-Miteaway application) and August 22nd (post-Miteaway application). In addition, colonies were treated with Fumagillan-B [9.1 g dissolved in 7.6 liters (two gallons) of sucrose or HFCS] in early October 2012 to control *N. apis* and *N. ceranae*, two common intestinal parasites. Entrance reducers were installed before the hives were ready for winterization.

All colonies were monitored weekly beginning on late October 2012. Notes were taken on the size of the clusters observed by counting the numbers of frames containing honey bees from the top of the hive in which it generally took no more than 10 seconds. Starting from November 2012, hives were supplemented either with crystallized HFCS or with granular sucrose mixed into a thick water paste. The food was placed on waxed paper on top of the frames inside the inner covers. Data were analyzed using SPSS Statistics (version 20.0).

Results

We found honey bee colonies in both control and neonicotinoid-treated groups progressed almost identically, and observed no acute morbidity or mortality in either group until the arrival of winter. In addition, neither the locations where the hives were set up nor the type of sugar (high-fructose corn syrup vs. sucrose) fed to honey bees was associated with the brood rearing or the occurrence of CCD (one-way ANOVA). Therefore data from 3 apiary locations and two types of sugar were pooled in the data analysis. As temperatures began to decrease in late October 2012, we observed a steady decrease of bee cluster size in both control and neonicotinoid-treated colonies. While such decline was quickly reversed in the control colonies in January 2013, the neonicotinoid-treated hives continued to decline (figure 1). As shown in table 1, the numbers of frames containing bees were not significantly different among the treatments from 10/27/2012 to 12/29/2012 (one-way ANOVA), but became statistically significant different from 1/5/2013 to 4/4/2013 (one-way ANOVA, $p < 0.0001$). At the end of the experiment on 4/4/2013, there were 5.3, 2.0, and 2.9 frames of bees in the control, imidacloprid, and clothianidin-treated hives, respectively. The diminishing cluster size in the neonicotinoid-treated colonies led to the loss of six of the twelve (50%) with symptoms resembling CCD, whereas only 1 of the 6 control colonies was lost exhibiting *Nosema ceranae* like symptoms, although we did not perform any test to confirm *Nosema* infection in this control hive. No similar *Nosema*-like symptoms were

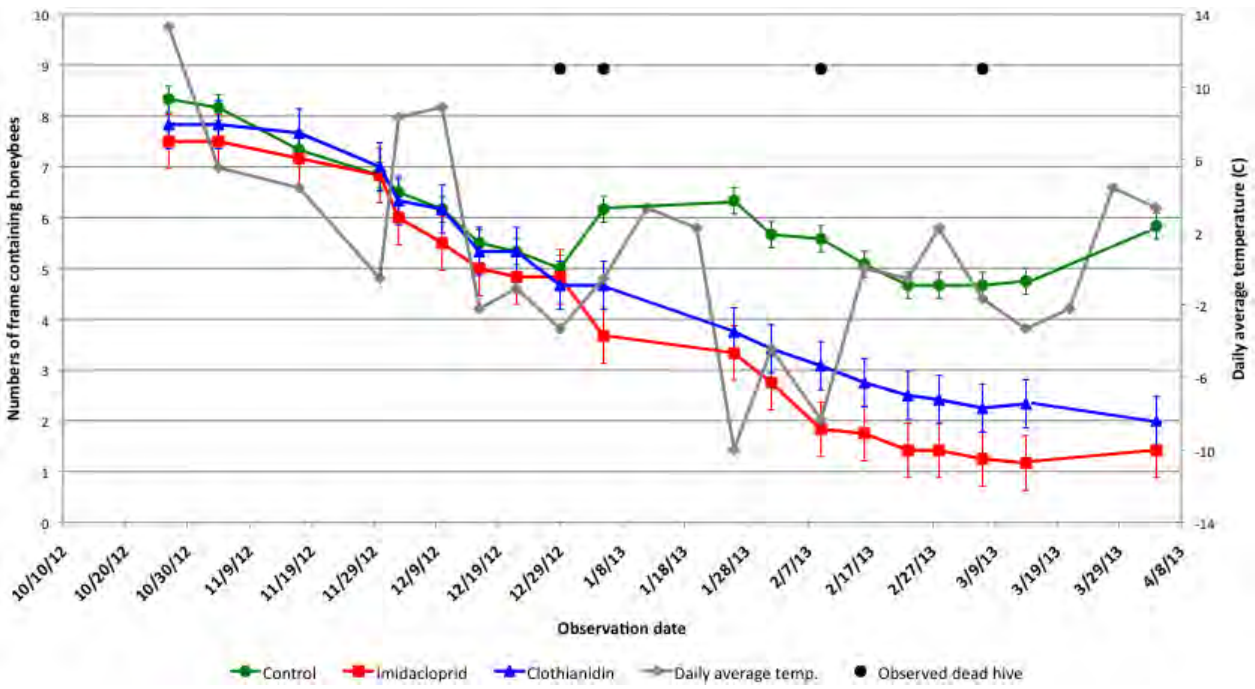

Figure 1. Average numbers of frame (standard deviations shown as error bars) containing honeybees for control-, imidacloprid-, and clothianidin-treated colonies and the corresponding daily average temperature at Worcester regional airport in Worcester MA recorded from October 2012 to April 2013. The daily average temperature readings were obtained from the NOAA website (http://cdo.ncdc.noaa.gov/qclcd/QCLCD).

Table 1. Field recording data from honey bee hives treated with control, imidacloprid, and clothianidin in sucrose water or high-fructose corn syrup (HFCS) from May 2012 to April 2013.

Treatment	Control		Imidacloprid		Clothianidin	
	Sucrose	HFCS	Sucrose	HFCS	Sucrose	HFCS
Honey bee hives	3	3	3	3	3	3
Average # of frame with bees (SD) Recorded from 10/27/2012 to 12/29/2012	6.3 (2)	6.8 (2)	6.0 (3)	6.3 (3)	6.6 (2)	6.3 (2)
Average # of frame with bees (SD) Recorded from 1/5/2013 to 4/4/2013	5.8 (1)	4.9 (3)	1.8 (2)	2.2 (2)	2.9 (2)	2.9 (2)
# of dead colony (%)	0 (0)	1 (33.3)	2 (66.7)	2 (66.7)	1 (33.3)	1 (33.3)
Date of dead colony observed		3/7/2013	1/5/2013 2/9/2013	1/5/2013 3/7/2013	1/5/2013	12/29/12
Average *Varroa* mite counts						
Before treatment (SD)	10 (6) [a]	11 (3) [a]	11 (2) [a]	10 (3) [a]	12 (2) [a]	9 (4) [a]
After treatment (SD)	2 (2) [b]	1 (1) [b]	1 (1) [b]	2 (1) [b]	1 (1) [b]	1 (1) [b]
Pooled Data [c]						
Honey bee hives	6		6		6	
Average # of frame with bees (SD) Recorded from 10/27/2012 to 12/29/2012	6.6 (2) [d]		6.1 (3) [d]		6.5 (2) [d]	
Average # of frame with bees (SD) Recorded from 1/5/2013 to 4/4/2013	5.3 (2) [e]		2.0 (2) [e]		2.9 (2) [e]	
# of dead colony (%)	1 (17)		4 (67)		2 (33)	
Average *Varroa* mite counts						
Before treatment (SD)	10 (4) [f]		12 (2) [f]		10 (3) [f]	
After treatment (SD)	2 (1) [f]		1 (1) [f]		1 (1) [f]	

[a] *Varroa* mite counts were not significantly different before Miteaway Quick strips treatment between sucrose and HFCS in control, imidacloprid, and neonicotinoid-treated hives (one-way ANOVA);
[b] *Varroa* mite counts were significantly different after Miteaway Quick strips treatment between sucrose and HFCS in control, imidacloprid, and neonicotinoid-treated hives (one-way ANOVA);
[c] Data from two sugar treatments were pooled for control, imidacloprid, and neonicotinoid-treated hives;
[d] Numbers of frame containing bees were not significantly different among control, imidacloprid, and neonicotinoid-treated hives during this period of time (one-way ANOVA);
[e] Numbers of frame containing bees were significantly different among control, imidacloprid, and neonicotinoid-treated hives during this period of time (one-way ANOVA, $p < 0.0001$);
[f] *Varroa* mite counts were significantly different before and after Miteaway Quick strips treatment in control, imidacloprid, and neonicotinoid-treated hives (paired t-test, $p < 0.0001$).

observed in the treated hives. Upon close examination of colonies in early April 2013, we found that the majority of bees in all neonicotinoid-treated colonies, regardless of whether they survived or not, had abandoned their hives during the course of winter. However, we observed a complete opposite phenomenon in the control colonies in which instead of abandonment, hives were re-populated quickly with new emerging bees. The honey bee clusters in the six surviving neonicotinoid-treated colonies were very small, and were either without queen bees, or had no brood.

We found no significant difference in the degree of *Varroa* mite infection between the control and neonicotinoid-treated colonies. The average mite counts were 10-12 per 150 bees in the control and neonicotinoid-treated colonies, respectively, as assessed in mid-August 2012 (table 1). We later reduced the mite counts in all colonies to 1-2 mites per 150 bees after the applications of Miteaway Quick strips, a commonly used medicinal treatments prior to the arrival of winter in which it significantly reduced mite counts from 10-12 to 1-2 mites per 150 bees, respectively, in control, imidacloprid, and neonicotinoid-treated hives (paired t-test, $p < 0.0001$). We also found that neonicotinoids do not appear to affect the quality of brood rearing during summer and fall (figure 2). The sealed brood counts for both control and neonicotinoid-treated colonies decreased significantly in parallel from July to September 2012 (Pearson, 2-tails, $p < 0.0001$). This decreasing (slope = −0.62) trend has been reported previously (Lu *et al*., 2012), and is consistent with a dirth of nectar that is common in the New England area during the summer, and is therefore independent of neonicotinoid exposure.

Discussion

The results from this study not only replicate findings from the previous study on imidacloprid and extend to clothianidin, but also reinforce the conclusion that sublethal exposure to neonicotinoids is likely the main culprit for the occurrence of CCD (Lu *et al*., 2012). The survival of 5 out of 6 control colonies in the same apiaries where the neonicotinoid-treated colonies were set up augment this conclusion. The observation of winter temperature modulating the severity of CCD associated

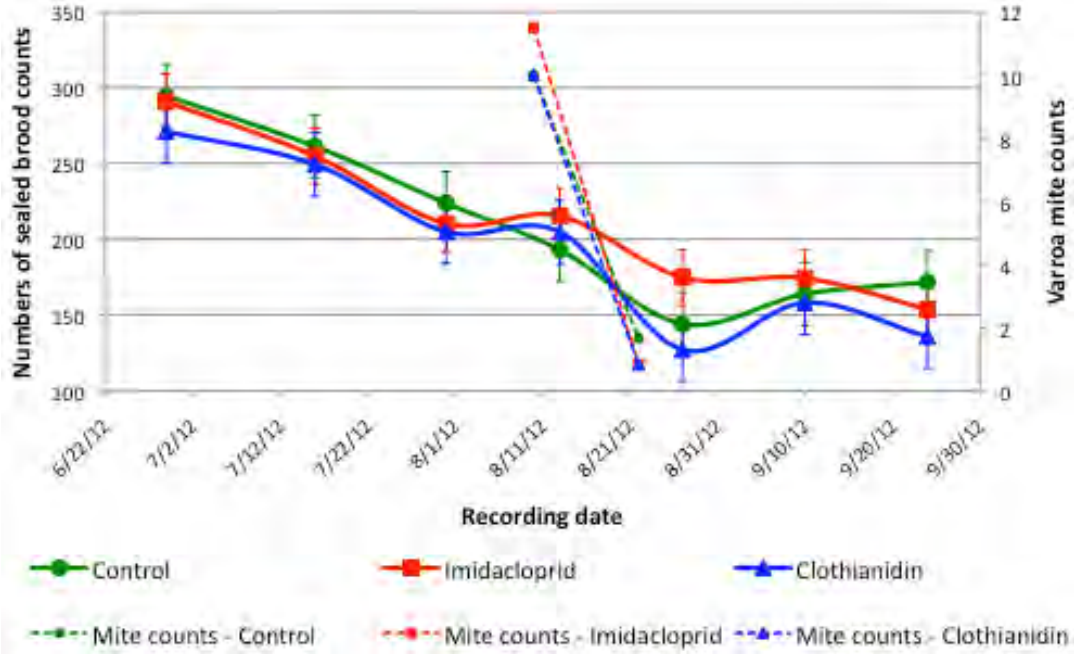

Figure 2. Average numbers of sealed brood count (standard deviations shown as error bars) for control-, imidacloprid-, and clothianidin-treated colonies during the dosing period (from 6/29/2012 to 9/24/2012), and the average numbers of *Varroa* mite counts recorded before and after Miteaway Quick strip treatment on 8/13/2012. Sealed brood counts were neither significantly different between sugars (one-way ANOVA) nor among treatments (one-way ANOVA). However, sealed brood counts were significantly decreased for all colonies from 6/29/2012 to 9/24/2012 (Pearson 2-tails, $p < 0.0001$).

with sub-lethal neonicotinoid exposure coincides with reports that CCD often occurs in the winter season. The modification of the sub-lethal effect of neonicotinoid by the severity of winter might be significant, and should not be overlooked in the evaluation of CCD epidemic. The previous study conducted during a colder winter reported 100% mortality of CCD in colonies treated with 0.1 ng/bee/day of imidacloprid (Lu *et al*., 2012), one-seventh of the dose used in the present study.

We found that chronic sub-lethal neonicotinoid exposures do not appear to compromise honey bees' immune resistance to pathogen infection in this study. This is in contrast to several earlier reports suggesting that the increased CCD mortality of honey bee colonies is due to reduced resistance toward common pathogens, such as increased susceptibility of *Nosema* infection, caused by neonicotinoid exposures (vanEngelsdorp *et al*., 2009; Alaux *et al*., 2010; Vidau *et al*., 2011; Pettis *et al*., 2012). The similar degree of *Varroa* mite infection in both control and neonicotinoid-treated colonies disagrees with the findings that CCD hives are often associated with significantly higher pathogen infestations than non-CCD hives exposures (vanEngelsdorp *et al*., 2009; Alaux *et al*., 2010; Vidau *et al*., 2011). In addition, a recent re-analysis of genomic data previously generated from RNA pools of CCD colonies has also excluded the association of pathogen infection and CCD (Tokarz *et al*., 2011). It is imperative to emphasize that while pathogen infections are common and serious diseases found in honey bees that often lead to colony death, the post-mortem examinations of the pathogen-caused dead colonies are vastly different to those suffered from CCD (Anderson and East, 2008; Lu *et al*., 2012). One of the defining symptomatic observations of CCD colonies is the emptiness of hives in which the amount of dead bees found inside the hives do not account for the total numbers of bees present prior to winter when they were alive (figure 3). On the contrary, when hives die in the winter due to pathogen infection, like the only control colony that died in the present study, tens of thousands of dead bees are typically found inside the hives (figure 4). The absence of dead bees in the neonicotinoid-treated colonies is remarkable and consistent with CCD symptoms.

Two critical questions remain to be answered in order to solve the CCD puzzle. First, why do neonicotinoid-treated colonies lose their ability to renew brood rearing toward the end of winter when temperatures began to rise? Considering that neonicotinoid-treated and control colonies had identical brood rearing performance prior to the arrival of winter (figure 1), the failure of neonicotinoid-treated colonies to resume brood rearing, in particular during the transition from winter to spring might be part of the interplay between sub-lethal neonicotinoid exposure and CCD. While it is true that the lack of brood rearing might simply be due to smaller surviving clusters during cold winter months, the surviving neonicotinoid-treated colonies never re-initiated the brood rearing into warm weather. We found that the severity of CCD caused by sub-lethal neonicotinoid exposures might be modulated by winter temperature. A colder and prolonged winter in 2010/2011 in central Massachusetts rendered a higher CCD mortality rate of 94% (Lu *et al*., 2012) than the current 50% in 2012/2013. Such disparity

Figure 3. Picture of the bottom board taken from one of the dead neonicotinoid-treated colonies on March 1st, 2013. The numbers of dead bees in the six dead CCD colonies ranged from 200-600 dead bees.

Figure 4. Picture of the bottom board taken from the only dead control colony on March 1st, 2013. The volume of dead bees was estimated to be 3.5 l using 1-L graduate cylinder using Atkins (1986) method.

might be due to the fact that the daily average temperature was lower in 63 of 91 days in the winter of 2010/2011 than of 2012/2013. The overall average temperature in the winter months was −3.8 °C (25 °F) in 2010/2011, approximately 2.78 °C (5 °F) lower than in 2012/2013.

Second and perhaps the foremost; why do honey bees vanish from neonicotinoid-treated colonies during the winter? It is striking and perplexing to observe the empty neonicotinoid-treated colonies because honey bees normally do not abandon their hives during the winter. This observation may suggest the impairment of honey bee neurological functions, specifically memory, cognition, or behavior, as the results from the chronic sublethal neonicotinoid exposure. Although the failure to initiate brood rearing and the vanishing of the worker caste in the neonicotinoid-treated colonies might be governed by completely different mechanisms, they suggest the possible involvement of cascading events prior to the occurrence of CCD. The findings from this study could be used to elucidate mechanisms by which sublethal neonicotinoid exposure impairs honey bees' ability to over winter with symptoms consistent with CCD.

We conclude that when honey bees were exposed to either imidacloprid or clothianidin at a dose of 0.73 ng/bee/day for 13 consecutive weeks from July to September 2012, six of twelve previously healthy neonicotinoid-treated colonies died and all progressed to exhibit CCD symptoms during the winter months. The survival of control colonies and the absence of CCD-like symptoms in the only dead control colony not only augment this conclusion but also support the finding that chronic

sub-lethal neonicotinoid exposure do not appear to compromise honey bees' immunity toward pathogen infection. The mechanisms by which sub-lethal neonicotinoid exposure caused honey bees to vanish from their hives during the winter months needs to be elucidated.

Acknowledgements

This study was generously supported by Wells Fargo Foundation and the Breck Fund established at the Harvard University Center for the Environment. The views expressed here are not necessarily those of Wells Fargo Foundation or the Breck Fund. We thank our friends, K. Desjardin, F. Jacobs, D. Lewcon, and J. Rogers who provided space to establish apiaries. We also thank M. Kapp and M. Chen for their assistance in the field study and the lab analysis.

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Received December 21, 2013. Accepted March 27, 2014.