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Influence of dinotefuran and clothianidin on a bee colony

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Abstract

Recently it has become a serious problem that honeybees suddenly vanish in their colony, which is referred to as a colony collapse disorder（CCD）. We have made it clear by the field experiments for about four months what effect neonicotinoid pesticides such as dinotefuran and clothianidin have on the occurrence of CCD. Eight colonies consisting of about ten-thousand honeybees in each colony were investigated under the practical beekeeping conditions in our apiary. In this study foods containing dinotefuran of 1 ppm to 10 ppm or clothianidin of 0.4 ppm to 4 ppm were fed into a beehive. Three levels of concentration were 10（high-conc.）, 50（middle-conc.）and 100（low-conc.）times lower than that in practical use. The changes of adult bees, brood and the pesticide intake in each colony were directly examined. They suggest that each colony with the pesticide administered collapses to nothing after passing through a state of CCD, the high-concentration pesticides seem to work as an acute toxicity and the low- and middle-concentration ones do as a chronic toxicity. CCD looks mysterious, but it is just one of situations where a colony dwindles to nothing. We have proposed a CCD occurrence mechanism based on our results. The NMR spectral analyses of dinotefuran and clothianidin in aqueous solution give the speculations that both are thermally stable under the heating condition of 50℃ ×24 hours and dinotefuran is radiationally stable under the ultraviolet-irradiation condition of 310 nm×50 W/m^2 but clothianidin is unstable.

《**Key words**》dinotefuran, clothianidin, neonicotinoid pesticide, colony, collapse

Ⅰ. Introduction

A phenomenon referred to as a colony collapse disorder（CCD）[1~4] causes extremely serious problems for not only bee-keeping but also yielding agricultural products through honeybee pollination, and furthermore sustaining ecosystem balance. The CCD differs from the general bee-behavior such as swarming in that nearly all the adult bees rapidly vanish while abandoning foods（honey, pollen）, brood and a queen.

Various theories on the cause of CCD have been till now proposed, such as a pesticide theory due to neonicotinoids[5~9], a mite and plague one due to Varroa mite and Israel acute paralysis virus（IAPV）[10~23], synergy-effect theory due to Nosema microspores and systemic pesticide such as a neonicotinoid[24, 25], besides an environmental change-related stress one[26, 27], a beekeeping- related stress one due to transportation and hard work, a nutrition stress one due to habitat loss[28], genetically modified（GM）crop one[29, 30], a radiation one due to a cellular phone, a multiple causes one[31~34], etc. At present, any of them has not been yet demonstrated scientifically with CCD reproduced directly and experimentally. It is difficult to reproduce CCD in a laboratory where the behavior of

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bees limited in number is observed because CCD is a phenomenon seen in a colony of bees which are eusocial insects. In this work, colonies which consist of enough honeybees (*Apis mellifera*) to behave as eusocial insects are prepared under a natural environment in an apiary. Dinotefuran and Clothianidin are widely used and well-known as a neonicotinoid pesticide in Japan mainly sprayed on rice field to exterminate stinkbugs that mark with spots on grains of rice and degrade them. We cannot find any reports about the influence of dinotefuran and clothianidin on a honeybee colony through a long-term field experiment in an apiary. In order to elucidate the long-term effect of these pesticides on a colony and the relationship between these pesticides and CCD, field experiments are carried out in an apiary based on our knowledge and experience in beekeeping.

The concentrations of pesticide administered to each experimental run are diluted 10-, 50- and 100-folds against the solution of commercial pesticide with a dilution factor recommended for exterminating stinkbugs in practical use. High-concentration pesticides were administered only in the beginning of experiment and after that they were never done in this study to clarify the influence of acute toxicity of the pesticides on a bee colony. Low and middle pesticides were administered every time to clarify the influence of chronic toxicity of them. In order to elucidate the change in strength of a colony with eusociality, this study will focus on the long-term behavior such as the change in the numbers of adult bees, brood and a queen in a colony, and the total intake of pesticide leading to the collapse of a colony during the administration of pesticide.

Generally, pesticides sprayed on the fields are diluted in water outdoors and the aqueous solutions are heated and irradiated with ultraviolet rays by the exposure to sunlight. In order to clarify the photolytic and pyrolytic properties of dinotefuran and clothianidin under the exposure to sunlight, their aqueous solutions heated at 50 °C and irradiated with ultraviolet rays are analyzed by the measurement of the proton NMR spectrum in this study.

Ⅱ. Experimental and Evaluation Methods

1. Experimental methods

【Field experiment of pesticide administration】

The pesticides were administered as foods of sugar syrup and pollen paste. Sugar syrup in a feeder and pollen paste on the combs were fed to each colony in a hive and they were exchanged for old ones every time. STARKLE MATE® with dinotefuran content of 10% by Mitsui Chemicals Aglo, Inc. in Tokyo (hereafter called "Starcklemate™") and DANTOTSU® with clothianidin content of 16% by Sumitomo Chemical Takeda Agro Company (hereafter called "Dantotsu™") were used in this study. They are representative neonicotinoid pesticides in Japan. The solutions of commercial pesticides with a dilution factor recommended for exterminating stinkbugs are as follows; a solution with a 1,000-fold dilution factor of a commercial concentration in sugar syrup for Starcklemate™ (dinotefuran of 100ppm in solution) and that with a 4,000-fold dilution factor for Dantotsu™ (clothianidin of 40 ppm in solution). Sugar syrup was made of an equal amount of sugar and water. The solution of pesticide administered to each experimental run are diluted 10-, 50- and 100-folds against the solution of commercial pesticide with a dilution factor recommended for exterminating stinkbugs in practical use. Now, we call the concentrations of 10-fold, 50-fold and 100-fold dilution "high", "middle" and "low", respectively. A solution with a 10-fold dilution factor of a recommended concentration of Starcle Mate™ is hereafter called S-high (10 ppm of dinotefuran) in RUN-2 and a solution with a 10-fold dilution factor of a recommended concentration of Dantotsu™ is hereafter called D-high (4 ppm of clothianidin) in RUN-5. Similarly, the solutions with the middle and low concentrations for Starcle Mate™ and Dantotsu™ are called S-middle (2 ppm of dinotefuran) (RUN-3), S-low (1 ppm of di-

notefuran) (RUN-4), D-middle (0.8 ppm of clothianidin) (RUN-6) and D-low (0.4 ppm of clothianidin) (RUN-7), respectively. The details of each experimental run are tabulated in Table 1. Pollen paste was prepared by kneading two parts of pollen with one part of sugar syrup containing the pesticide, where the pollen was prepared by mixing the pollen substitute "Feed-Bee®" with pure pollen at a ratio of 1:1. Pictures of both sides of all the combs, a feeder, the inside, the outside of a hive, etc. were taken with a digital camera at weekly intervals (rarely at two-weekly intervals). Pictures of circumstances around the entrance of a hive were taken with a digital camera with a half-hourly–interval timer monitoring the activities of honeybees such as carrying the dead bees out of a hive and the like.

Experiments were conducted in an apiary, where crop-dusting is controllable and honeybees could freely visit flowers in the field and avoid taking the foods with pesticide in their hive if they prefer natural nectar, pollen and water in the surrounding fields. In order to count the number of adult bees as correctly as possible, experiments were conducted before foraging bees went out of the hive in the early morning except on rainy days, fundamentally at weekly intervals (rarely, at two-weekly intervals). Eight standard hives with the entrance to the east were arranged on a hill from south to north. Each was composed of six combs and one feeder of sugar syrup. In order to prevent honeybees from swarming, a comb foundation was newly added in the hive of each blank run (control) when necessary. We have conducted a series of experiments since July 18th in 2010 for about four months when there were not many flowers in bloom and it is the less-swarming season of honeybees in Japan.

Table 1 Outline of foods (sugar syrup, pollen paste) on each experimental run

RUN No.	Administered pesticide	A Dilution of commercial product [1]	A Dilution of the reference solution [2]	Content of pesticide [3]	Notation [4]	Note [5]
RUN01	No pesticide			0 ppm	B-1 (Blank run)	(control)
RUN02	Starckle™ (dinotefuran 10%)	10,000-fold dilution	10	10 ppm	S^{10}_{10000}	S-high
RUN03	Starckle™ (dinotefuran 10%)	50,000-fold dilution	50	2 ppm	S^{50}_{50000}	S-middle
RUN04	Starckle™ (dinotefuran 10%)	100,000-fold dilution	100	1 ppm	S^{100}_{100000}	S-low
RUN05	Dantotsu™ (clotianidin 16%)	40,000-fold dilution	10	4 ppm	D^{10}_{40000}	D-high
RUN06	Dantotsu™ (clotianidin 16%)	200,000-fold dilution	50	0.8 ppm	D^{50}_{200000}	D-middle
RUN07	Dantotsu™ (clotianidin 16%)	400,000-fold dilution	100	0.4 ppm	D^{100}_{400000}	D-low
RUN08	No pesticide			0 ppm	B-2 (Blank run)	(control)

[1] Dilution of commercial pesticide means that a commercial pesticide is diluted with sugar syrup up to a given dilution factor. For example, in RUN02 a commercial Starckle™ containing dinotefuran of 10% is diluted with 10,000 parts of sugar syrup, where the solution in RUN02 contains dinotefuran of 10 ppm. The concentration of a pesticidal constituent included in a commercial pesticide are dinotefuran of 10% in Starcklemate™ and clothianidin of 16% in Dantotsu™, respectively.

[2] Dilution of the reference solution represents a dilution factor diluting the reference solution which is recommended as a concentration of extermination of stinkbugs, where the reference solution of Starcklemate™ and Dantotsu™ have a 1,000-fold dilution of a commercial product (dinotefuran of 100 ppm in solution) and a 4,000-fold dilution of one (clothianidin of 40 ppm in solution), respectively.

[3] Content of pesticide represents the content of main constituent of pesticide administered to each run. For example, a 10 ppm of dinotefuran is administered to RUN02, which is included in a 10,000-fold diluted Starcklemate™.

[4] B-1 and B-2 represent blank runs. X and Y in S^X_Y and D^X_Y represent the X-fold dilution of the reference solution and the Y-fold dilution of the commercial product, respectively, and the S and D represent Starcklemate™ and Dantotsu™ , respectively.

[5] High conc. (concentration), middle conc., and low conc. means a 10-fold dilution of the reference solution , a 50-fold one and a 100-fold one in this paper, respectively.

【Nuclear Magnetic Resonance (NMR) measurement of dinotefuran and clothianidin】

Dinoterufan (standard) and clothianidin (99.5%) were purchased from Kanto chemical (Japan) and Dr. Ehrenstorfer GmbH (Germany), respectively. These pesticides were used without further purification. Samples were dissolved in D_2O containing 0.3% trimethylsilyl propanoic acid as a standard. Decomposition by heating at 50℃ for 24 hours and ultraviolet (UV) light irradiation for 30 min at 310 nm × 50 W/m^2 was investigated by 1H-NMR measurements, where the amount of UV light irradiation is equivalent to that of about 6.5-days UV radiation from the sun in Tsukuba city. UV light may be somewhat decreased in intensity because it is irradiated on a sample through a glass container. UV light irradiation was performed on Funakoshi NTM-10 trans-illuminator. NMR spectra were obtained by JEOL ECS-400 spectrometer at room temperature.

2. Evaluation methods

The change in the numbers of adult bees and brood in each colony was directly examined through a long period of days in this work because the change in the weight of a hive contained all the changes in the weight of honey, pollen and others in addition to honey bees and brood.

The numbers of adult bees and brood (capped brood and visible larvae) on a comb were counted and summed up in a hive. The number of adult bees on a comb was directly counted on a photo when less than several hundreds; it was indirectly counted when more than several hundreds by use of the reference photos which were directly counted beforehand. The sum total on all combs in a hive was used as the number of adult bees for each run. The number of brood was evaluated on a photo by the ratio of the area occupied with brood to the whole surface on one side of a comb. The sum total of the area ratios on all combs in a hive was expressed as the number of brood for each run in this study.

These numbers were double-checked by two persons.

The consumption of foods (sugar syrup, pollen paste) by honeybees and the number of dead bees were estimated from photos and visual measurements at every experiment. The intake of pesticide was calculated from the consumption of foods. The total intake of pesticide leading to the collapse of a colony is converted into the pesticide solution with a concentration of a commercial product (STARKLE MATE®, DANTOTSU®) from the consumption of sugar syrup or pollen paste.

3. Definition of normalized number

To compensate for a difference in initial population among runs and that in seasonal fluctuation of bee population, a relative change in the number of adult bees is newly defined by the following Equation (1)

Normalized number of adult bees

$$= (n_{ij} / n_{i0})/(n_{Bj} / n_{B0}) \quad (1)$$

Where,

n_{ij} = the number of adult bees in RUN i after the elapse of j days,

n_{i0} = the initial number of adult bees in RUN i at the start of experiment,

n_{Bj} = the number of adult bees in blank run after the elapse of j days,

n_{B0} = the initial number of adult bees in blank run at the start of experiment,

where the arithmetic mean number of RUN-1 and RUN-8 was used as the number of adult bees in blank run in Equation (1).

A period of brood is considerably shorter than that of an adult bee and not always contemporary with each other colony. Therefore, the change in the number of brood was evaluated without normalization.

Ⅲ. Results and Discussion

1. Change in the number of adult bees

Table 2 shows the change in the number of total adult bees in a hive with the elapsed days for each run. Figure 1 shows the change in the number of total adult bees normalized by Equation (1). The fol-

Table 2 Change in number of total adult bees with elapsed days for each run

Start of the experiment after the adjustment on initial number of total adult bees

Date in 2010	Elapsed days	RUN 1 *control*	RUN 2 *S-high*	RUN 3 *S-middle*	RUN 4 *S-low*	RUN 5 *D-high*	RUN 6 *D-middle*	RUN 7 *D-low*	RUN 8 *control*	Average of Blanks
(Pesticide)		Blank 1	Starcklemate™	Starcklemate™	Starcklemate™	Dantotsu™	Dantotsu™	Dantotsu™	Blank 2	Blank 1 & 2
(Dilution[1])		No pesticide	10,000-fold[1]	50,000-fold[1]	100,000-fold[1]	40,000-fold[1]	200,000-fold[1]	400,000-fold[1]	No pesticide	No pesticide
July 18	0	8950	11700	12720	10400	12880	11600	13400	10560	9755
July 23	5	11700	5450	5240	7900	5100	7800	11900	11400	11550
July 30	12	11850	(3900)	7250	8750	(1770)	8900	12100	11800	11825
August 8	21	11100	(2550)	1235	9500	(1775)	4060	10100	12400	11750
August 13	26	11400	(1450)	940	8500	(1530)	[70]	9900	11800	11600
August 21	34	8900	(861)	325	4750	(640)	[275]	6300	10700	9800
August 26	39	9800	(980	200	5150	(890)	[36]	4340	9400	9600
September 5	49	9650	(760)	[178]	4590	(830)	[0]	[1840]	6370	8010
September 11	55	10600	(666)	[110]	3550	(810)		[1180]	7450	9025
September 17	61	11150	(264)	[0]	3740	(730)		[975]	6150	8650
September 24	68	12300[2]	(470)		1395	(895)		[150]	7680[2]	9990
October 10	84	12300[2]	(415)		0	(740)		[0]	7680[2]	9990
October 30	104	12300[2]	(0)			(285)			7680[2]	9990
November 21	126	12300[2]				[(0)]			7680[2]	9990

[1] This shows a dilution factor of a commercial product. [2] The numbers of adult bees on the elapsed of 68 days in RUN 1 & 8 were substituted for that after that.
(Note) Parentheses ( ) show a state that foods (sugar syrup, pollen paste) without a pesticide were fed into a colony after the elapse of 12 days instead of foods with a pesticide. Brackets [ ] show a state that a queen had been lost. The average between RUN 1 & 8 was used as the number in blank run in calculation of normalized number. Starcklemate™ contains a dinotefuran content of 10% and Dantotsu™ contains a clothianidin content of 16%. Less than ten heads are expressed as zero.

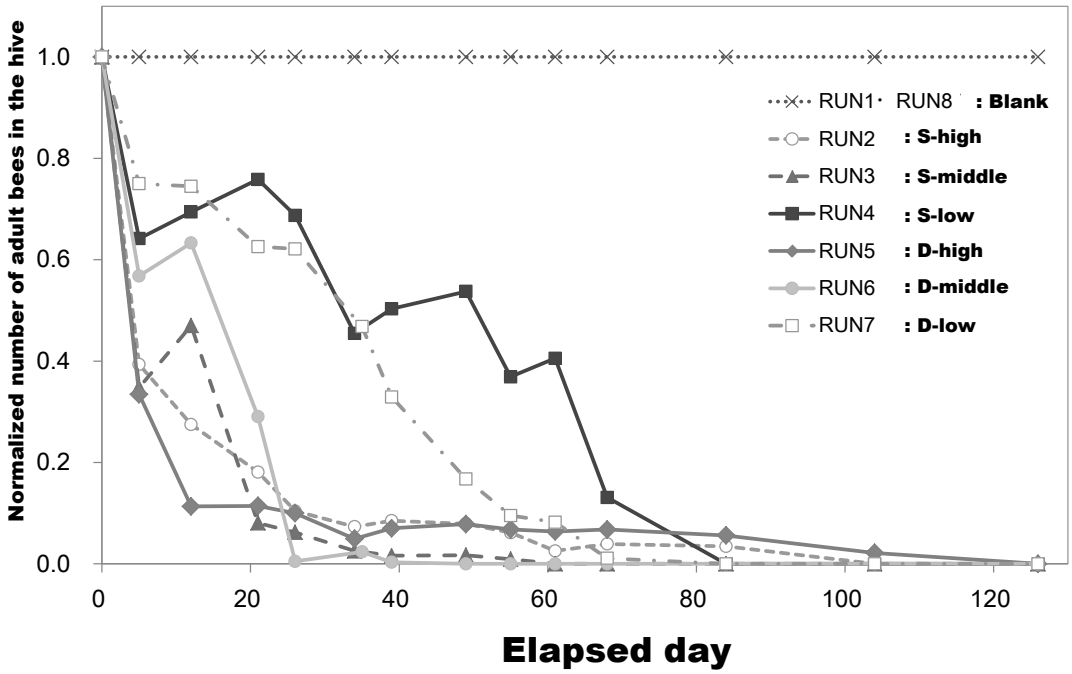

Figure 1 Normalized number of adult bees in the hive with the elapsed days

lowing results can be obtained: After the administration of the pesticides (dinotefuran, clothianidin), the number of adult bees rapidly dwindled and the colony became extinct afterwards. A queen bee did not disappear until adult bees became few. It is confirmed from photos that brood and foods existed at the point of queen's loss. Wax-moth larvae did not exist in a hive while adult bees decreased in number to nothing and for a while after the complete collapse of a colony.

In S-high (RUN-2) and D-high (RUN-5), adult bees were killed on the instant just after the administration of pesticide. The foods with a high-concentration pesticide were fed to a colony only in the first stage of experiment and they were replaced by foods without pesticide twelve days later. A great number of dead bees occurred in and around the hive for twelve days after the administration of pesticide. In S-high, some dead bees were found three weeks later but afterwards became a few. In D-high, a few dead bees were found three weeks later and afterwards. The colony became extinct fifteen weeks later in S-high and eighteen weeks later in D-high. A queen existed until the number of adult bees dwindled down to zero in S-high and D-high.

In S-middle (RUN-3) and D-middle (RUN-6), the number of adult bees decreased to nothing seven weeks later in D-middle and about nine weeks later in S-middle. A queen existed until the number of adult bees dwindled down to 1.4 percent of the initial number in S-middle and 0.6 percent in D-middle. A number of dead bees occurred only in the early period after administration but they almost never occurred in S-middle and D-middle afterwards.

In S-low (RUN-4) and D-low (RUN-7), the number of adult bees decreased to nothing twelve weeks later in the same period of time. A queen existed until the number of adult bees dwindled down to zero in S-low and about 14 percent of the initial number in D-low. Dead bees almost never occurred after administration.

2. Change in the number of brood

Table 3 and Figure 2 show the change in the number of total brood in a hive with the elapsed days for each run. The following results can be obtained from them: The number of brood sharply decreased after the first pesticide administration while taking a peak in some cases about five weeks later. Taking a peak was caused by stimulation in egg-laying of a queen due to the sharp decrease in the number of brood. This suggests that a pesticide has some effect on egg-laying and hardly any effect on eggs and larvae. The decrement in brood roughly suggests that the higher concentration of pesticide leads to the more serious egg-laying impediment of a queen. At the elapse of twelve days, the egg-laying capacity of a queen rapidly declines and is kept low afterwards, independently of the pesticide concentration, though a high-concentration pesticide was stopped while foods without pesticide being fed.

From the long-term observational results of brood, a colony with the pesticide administered collapses to nothing after passing through a state of CCD as supported in a new article titled "*in situ* replication of honeybee colony collapse disorder"[35] due to neonicotinoid pesticide (imidacloprid) which was published just after submitting this article to this journal.

3. Total intake of pesticide leading to the collapse of a colony

Table 4 and Figure 3 show the total intake of pesticide. The following results can be obtained from them: In the case of S-high (RUN-2) and D-high (RUN-5), a colony resulted in a collapse even when a high-concentration pesticide was administered to a colony only in the first stage of experiment and afterwards foods with high-concentration pesticide was stopped and replaced by those without pesticide. This suggests that a colony probably collapses due to acute toxicity in high pesticide concentrations which is one tenth the concentration to exterminate stinkbugs in practical use. If the rough assumption is made that five hundred honeybees a colony newly

Table 3 Change in number of total brood in a hive with elapsed days for each run

Start of the experiment after the adjustment on the initial number of broods

Date in 2010	Elapsed Days	RUN 1 *control*	RUN 2 *S-high*	RUN 3 *S-midle*	RUN 4 *S-low*	RUN 5 *D-high*	RUN 6 *D-middle*	RUN 7 *D-low*	RUN 8 *Control*	Average of Blanks
(Pesticide)		Blank 1	Starcklemate™	Starcklemate™	Starcklemate™	Dantotsu™	Dantotsu™	Dantotsu™	Blank 2	Blank 1 & 2
(Dilution[1])		No pesticide	10,000-fold[1]	50,000-fold[1]	100,000-fold[1]	40,000-fold[1]	200,000-fold[1]	400,000-fold[1]	No pesticide	
July 18	0	5.3	7.05	7.2	3.9	7.6	1.5	2	6.96	6.13
July 23	5	5.25	4.45	3.95	4.05	2.6	1.4	2.1	4.45	4.85
July 30	12	3.7	(0.8)	1.4	1.35	(0.25)	0.05	0.05	3.5	3.6
August 8	21	2.5	(0.4)	0	0.05	(0.2)	0.15	0.3	3.45	2.975
August 13	26	2	(0.4)	0	0.1	(0.05)	[0.2]	0.35	2.95	2.475
August 21	34	2.55	(0.4)	0.07	0.6	(0.3)	[0.006]	0.8	2.8	2.675
August 26	39	2.5	(0.15)	0.1	0.25	(0.4)	[0]	0.235	2.05	2.275
September 5	49	1.4	(0.05)	[0.039]	0.036	(0.05)	[0]	[0.049]	2.1	1.75
September 11	55	1.5	(0.065)	[0.065]	0.008	(0.098)		[0]	2.6	2.05
September 17	61	1.9	(0.042)	[0.042]	0.005	(0.099)		[0]	3	2.45
September 24	68	3.85[2]	(0.016)		0	(0.045)		[0]	4.4	4.125[2]
October 10	84	3.85[2]	(0.06)		0	(0.141)		[0]	4.4	4.125[2]
October 30	104	3.85[2]	(0)			(0.026)			4.4	4.125[2]

[1] Dilution shows a dilution factor of a commercial product. [2] The numbers of brood on the elapsed of 68 days in RUN 1 & 8 were substituted for that after that.

(Note) Parentheses ( ) show a state that foods (sugar syrup, pollen paste) without a pesticide were fed into a colony after the elapse of 12 days instead of foods with a pesticide.

Brackets [ ] show a state that a queen had been lost. Where Starcklemate™ contains a dinotefuran content of 10% and Dantotsu™ contains a clothianidin content of 16%.

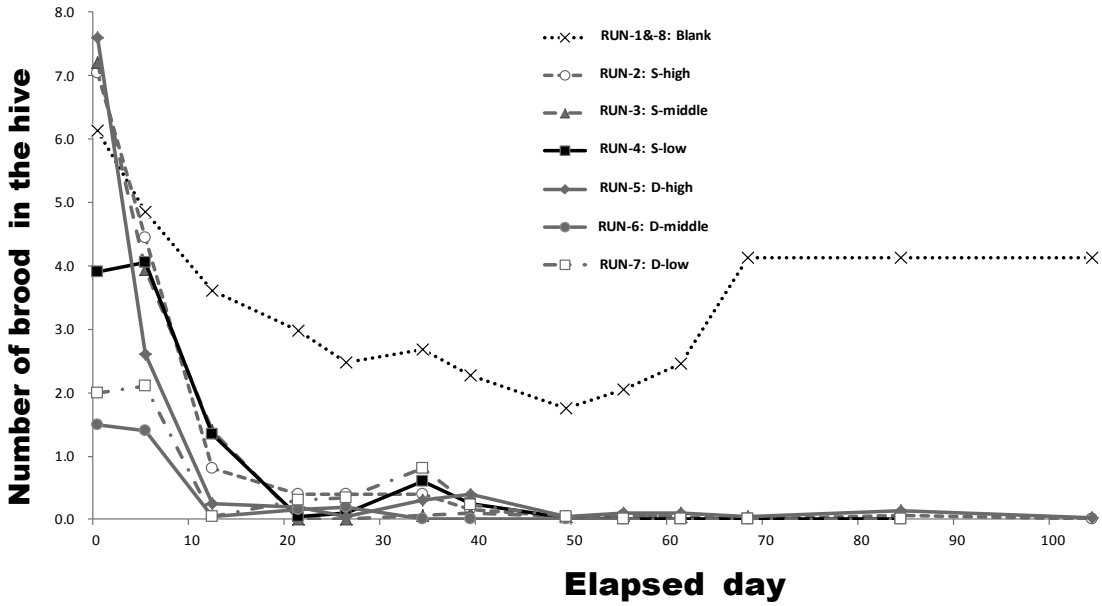

Figure 2 Change in the number of brood expressed by the number of combs occupied by brood in the hive with the elapsed days

develop from larvae into imagoes while influenced by foods with the pesticide fed and stored in the hive, the lethal dose of dinotefuran can be estimated to be 0.1072 μg/bee for S-high (RUN-2), 0.2434 μg/bee for S-middle (RUN-3) and 0.1903 μg/bee for S-low (RUN-4), respectively. Similarly, the lethal dose of clothianidin can be estimated to be 0.0360 μg/bee for D-high (RUN-5), 0.1150 μg/bee for D-middle (RUN-6) and 0.0706 μg/bee for D-low (RUN-7), respectively. Iwasa et al.[36] reported that the LD_{50} values of dinotefuran and clothianidin are 0.0750 μg/bee and 0.0218 μg/bee, respectively. The report by Iwasa et al.[36] roughly supports the results of this work.

On the other hand, a colony probably collapses due to chronic toxicity in the middle and low pesticide concentrations as already concerned about possible chronic problems caused by long-term pesticide exposure in nectar[4]. Because there is little difference of the total pesticide intake leading to the collapse of a colony between S-middle (RUN-3) and S-low (RUN-4) and similarly little difference of that between D-middle (RUN-6) and D-low (RUN-7). This suggests that the pesticide may be little-metabolized and accumulated in the body tissues of bees and then a colony probably collapses due to the chronic toxicity when the accumulated pesticide pass a certain threshold.

On closer investigation, the total intake of pesticide leading to the collapse in S-middle or S-low is about 150 percent of that in S-high. Similarly, the total intake of pesticide in D-middle or D-low is about 150 percent of that in D-high. From the above it can be suggested that the total intake of pesticide leading to the collapse in the low or the middle (chronic toxicity) is about 150 percent of that in the high (acute toxicity).

The total intake of dinotefuran (Starcklemate™) leading to the collapse of a colony is almost four times as much as that of clothianidin (Dantotsu™) in the concentration of commercial product, independent of the pesticide concentration; that is, S-high/D-high ≒

Table 4 Total intake of pesticide for each run calculated from the intake of foods

Fiducial concentration	Total intake of pesticide	RUN 1	RUN 2	RUN 3	RUN 4	RUN 5	RUN 6	RUN 7	RUN 8
		Control	S-high	S-middle	S-low	D-high	D-middle	D-low	Control
Reference solution[a] [g]	from sugar syrup [g]	0	63.3	99.8	95	63.2	98.8	93.2	0
	from pollen paste [g]	0	5	5.4	4.7	5.2	5.2	4.8	0
	from both foods [g]	0	68.3	105.2	99.7	63.4	104	98	0
Commercial product[b] [mg]	from sugar syrup [mg]	0	63.3	99.8	95	15.8	24.7	23.3	0
	from pollen paste [mg]	0	5	5.4	4.7	1.3	1.3	1.2	0
	from both foods [mg]	0	68.3	105.2	99.7	17.1	26	24.5	0
Active ingredient[c] [mg]	from sugar syrup [mg]	0	6.33	9.98	9.5	2.53	3.95	3.72	0
	from pollen paste [mg]	0	0.5	0.54	0.47	0.2	0.2	0.19	0
	from both foods [mg]	0	6.83	10.52	9.97	2.73	4.15	3.91	0
		no pesiticide	dinotefuran			clotianidin			no pesticide

[a] Total intake of pesticide solution converted into the reference solution with a concentration to exterminate stinkbugs
[b] Total intake of pesticide solution with the concentration which is converted into the concentration of commercial product
[c] Total intake of pesticide converted into the amount of an active ingredient which is dinotefuran for RUN-2, -3 and -4 or clotianidin for RUN-5, -6 and -7
(Note) The total intake of pesticide which was converted into the pesticide solution with a concentration of a commercial product (Starcklemate™, Dantotsu™) from the comsumption of sugar syrup or pollen paste.
Where Starcklemate™ contains a dinotefuran content of 10% and Dantotsu™ contains a clotianidin content of 16%.

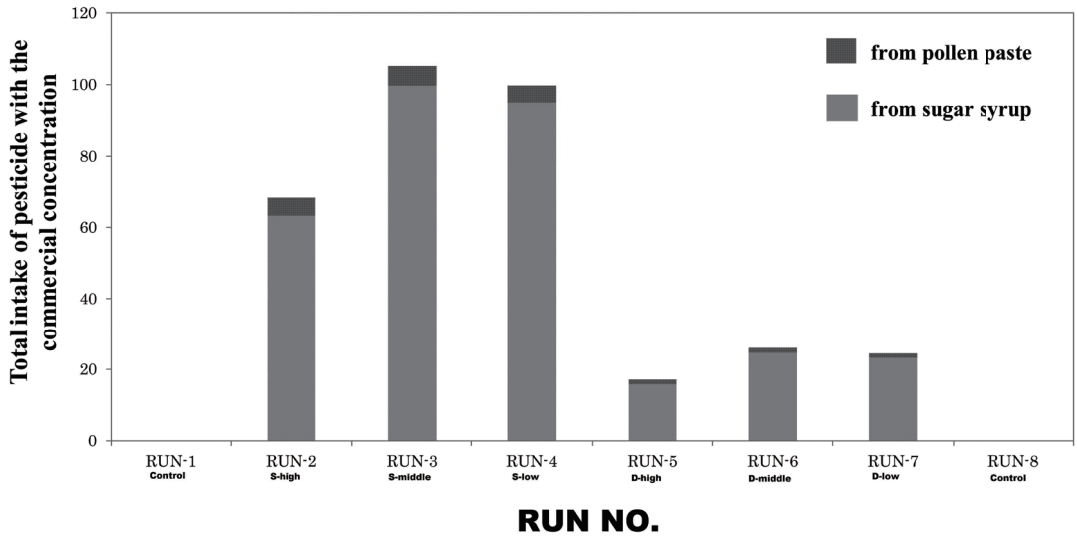

Figure 3 Total intake of pesticide with a converted concentration into that of commercial product for each run

4; S-middle/D-middle ≒ 4; S-low/D-low ≒ 4.1. The ratio between the dilution factor to make the solution to exterminate stinkbugs of clothianidin and that of dinotefuran is 4000:1000=4:1. Considering that each of them has the same insecticidal activity against a stinkbug, Starcklemate™ seems to have almost the same insecticidal activity against a honeybee as Dantotsu™.

When converting the food consumption into the amount of active ingredient which is pure dinotefuran or clothianidin, the ratios of total intake of pesticides in high, middle and low concentration are 2.50 for S-high/D-high, 2.53 for S-middle/D-middle and 2.55 for S-low/D-low, respectively. From the above, the insecticidal activity of clothianidin is about 2.5 times as strong as that of dinotefuran, while slightly increasing with decrease in pesticide concentration.

4. Photolytic and pyrolytic properties of dinotefuran and clothianidin on the assumption that an aqueous solution of pesticide is exposed to sunlight

Figures 4 and 5 show the measured results of the proton NMR spectra for dinotefuran and clothianidin, respectively. These NMR spectral analyses give the following speculations:

1) Dinotefuran and clothianidin is not decomposed at 50 °C.

2) Dinotefuran is ultraviolet-stable because of lack of chromophore under the conditions of radiation intensity (RI) = 50 W/m^2, wavelength (WL) = 310 nm and radiation time (RT) = 0.5 hrs equivalent to about 6.5-days UV radiation amount from the sun in Tsukuba city. This is somewhat different from the underwater photolysis testing results of dinotefuran with a xenon arc lamp under the conditions of RI = 400−416 W/m^2, WL = 300−800 nm and RT = 3.8 hrs[37] equivalent to about 400-days UV radiation amount from the sun in Tsukuba city. The difference may come from the amount of UV light irradiation. As a pesticide is expected to be photo-decomposed as soon as possible after sprayed, about 400-days UV radiation amount from the sun seems to be too much in comparison with the half-life of 180 days regulated by law[38].

3) Clothianidin is decomposed by ultraviolet rays under the same conditions as dinotefuran because it has a thiazole ring absorbing ultraviolet rays. This is approximately similar to the underwater photolysis

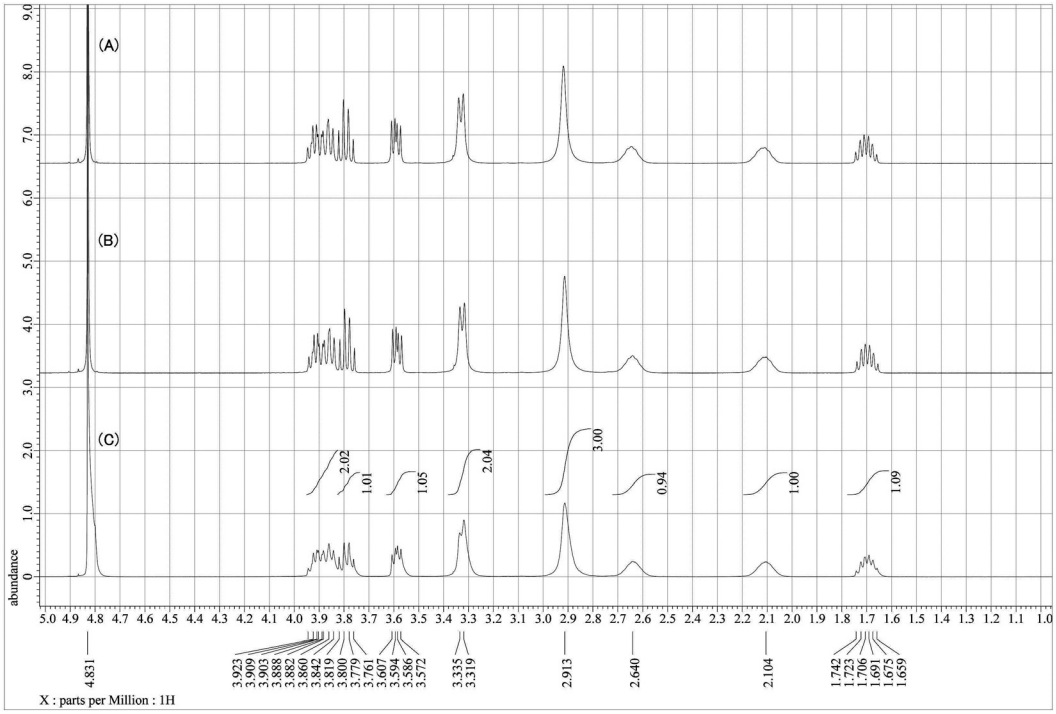

Figure 4 NMR spectra of dinotefuran in D_2O (A) without any treatment, (B) after heating at 50 ℃ for 24 hours, and (C) after UV light irradiation for 30 min.

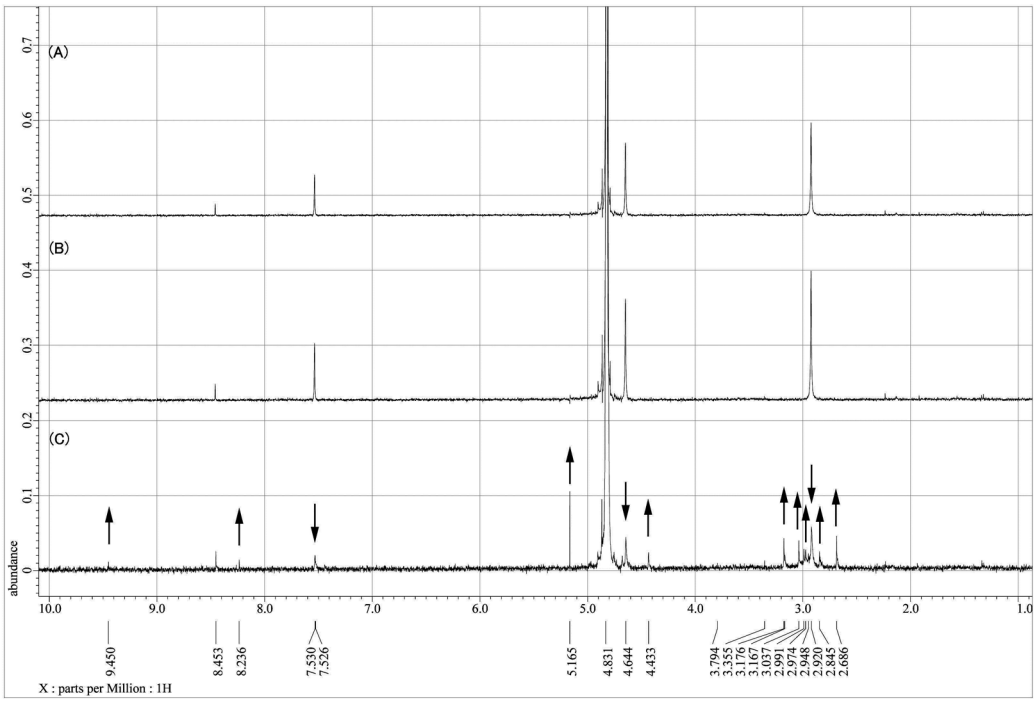

Figure 5 NMR spectra of clothianidin in D_2O (A) without any treatment, (B) after heating at 50℃ for 24 hours, and (C) after UV light irradiation for 30 min. The increased and decreased signals were shown in the figure as up and downward arrows.

testing results of clothianidin with a xenon arc lamp under the conditions of RI=18 W/m^2, WL=360−480 nm and RT=40−42 min[39] equivalent to about 3-days UV radiation amount from the sun in Tsukuba city. The decomposition products by ultraviolet rays seem to be extremely diverse because the skeleton of nitroguanidine is biodegradable under the anaerobic condition. The specification and toxicity of the decomposition products are unexamined.

5. Rational mechanism of CCD occurrence

Dinotefuran and clothianidin can lead to the collapse of a bee colony, judging from the following experimental findings in this study: 1) Dinotefuran and clothianidin are probably little-metabolized and mostly accumulated chronically in the body tissues of bees and work as an chronic toxicity in low and middle concentrations. 2) A high-concentration pesticide seems to work as an acute toxicity just by one dose judging from the total pesticide intake till the collapse of a colony, which is less than that of low or middle concentration pesticide, and the state of dead bees. 3) As a period of brood is very short, the low-concentration pesticide does not much affect the brood but does a queen having a long lifetime and results in the inhibition of her egg-laying. 4) Both dinotefuran and clothianidin are thermally stable. And dinotefuran is stable under ultraviolet irradiation but clothianidin is unstable (quite susceptible to deterioration from ultraviolet light). 5) Starcklemate™ (dinotefuran) seems to have almost the same insecticidal activity against a honeybee as Dantotsu™ (clothianidin) when they are prepared to have the same insecticidal activity against a stinkbug.

We can infer the following plausible mechanism of CCD occurrence as an example from the findings mentioned above: Figure 6 shows the schematic diagram of CCD occurrence mechanism due to neonicotinoid pesticides. Considering the fact that the concentration of pesticide sprayed on fields is at least ten times higher than that in this study, and under the assumption that a low-concentration pesticide diluted in water is stable under the sunlight and the toxicity does not change for a long time, a colony can be presumed to collapse as bellow:

Foraging bees are killed instantly on the spot where a pesticide is directly sprayed. The death of many foraging bees leads to the conversion of house bees into foraging bees, and as a result a lack of house bees and imbalance of colony composition. When foraging bees take water, nectar or pollen containing pesticide in high concentrations, they are killed instantly near the sprayed spot. Judging from the fact that about 5 ppm of clothianidin was detected in the water near the rice paddy[40], the above assumption seems to be plausible. When in middle concentrations, some are killed instantly near the sprayed spot and others come back to their hive and then soon die. In this case many dead bees are found near their hive. On the other hand, the sprayed pesticide is diluted with water in a rice paddy or rain, or the toxicity of nectar has been diluted by new nectar flowing out in a flower. In such cases foraging bees are scarcely killed on the spot. And ingesting water, nectar and pollen with a pesticide in low concentrations, foraging bees carry the low toxic ones back to their hive. The low toxic ones are ingested by house bees, brood and the queen, or stored in combs as honey and bee bread, and then the pesticide accumulated in the body of bees passes a certain threshold of toxicity. When the brood taking the pesticide become foraging bees, they cannot come back to their hive because of being disoriented or becoming exhausted due to chronic toxicity as suggested in the recent article on homing failure in foraging honeybees[41]. The egg-laying capacity of a queen declines through ingesting the low toxic ones but a queen remains until the collapse. The imbalance of colony composition also causes the decrease in the egg-laying activity of a queen and finally leads to a collapse of the colony with a queen remaining. Even if a colony appear to be vigorous before wintering after it has been influenced by low toxicity in autumn, it probably fails in wintering due

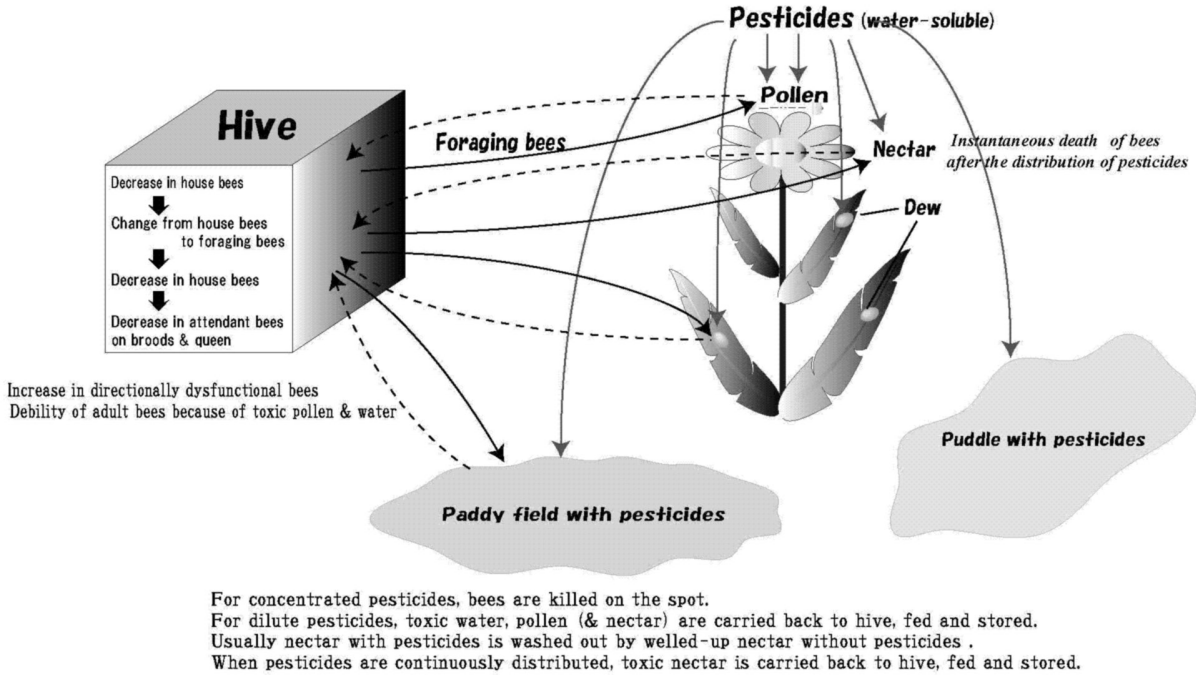

Figure 6 Schematic diagram of CCD occurrence mechanisms due to neonicotinoid pesticides

to chronic toxicity. Even when a colony does not collapse and looks active, a neonicotinoid pesticide causes an egg-laying impediment of a queen and a decrease in immune strength of bees leading to the infestation of mites in a colony.

Ⅳ. Conclusion

A colony rapidly dwindled after the administration of dinotefuran or clothianidin and finally became extinct after taking on an aspect of CCD. That is, a queen bee did not disappear until adult bees became few and brood and foods existed in the colony at the point in time when a queen disappeared. Wax-moth larvae did not exist for some time after the extinction of colony. This means that the CCD is just one of situations where a colony dwindles away to nothing although it may look mysterious. These strongly suggest that the neonicotinoid pesticides such as dinotefuran and clothianidin can most probably causes CCD whose mechanism is proposed as follows: In supposing that a pesticide is sprayed and diluted in water of a rice paddy or an orchard and its concentration becomes low, the low-concentration pesticide carried by foraging bees continues to affect a colony for a long time and finally leads to a collapse of a colony or the failure in wintering. Even if a colony does not collapse and looks active, it causes an egg-laying impediment of a queen and a decrease in immune strength of bees leading to the infestation of mites in a colony.

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The authors have received valuable advices and informative collaboration from Mr. Seita Fujiwara, Dr. Yasuhiro Yamada and people involved in bee-keeping. And NMR analyses were performed with the support of the Advanced Science Research Center of Kanazawa University. This study was supported from a fund for research on bees granted by Yamada Apiary.

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要約

蜂崩壊症候群（CCD）と呼ばれる現象は養蜂や農業のみならず、生態系の危機へ繋がる深刻な問題である。病原体説や農薬説など様々なCCD原因説が提案されているが、決定的な結論は出ていない。これまでCCD原因解明のために、限定された条件下での実験やCCD発生後の巣箱内の病原体の分析等が行われてきたが、CCD発生過程の長期現場実験は殆ど行われていない。欧米ではネオニコチノイド系農薬を状況証拠から使用禁止した国も多いが、日本では科学的根拠が確定されていないため禁止に至っていない。そこで、日本で広く使われているジノテフランとクロチアニジンの長期投与実験を行い、その間の蜂数や蜂児数の変化および農薬摂取量を追跡し、蜂群がCCDの状態を経由して消滅に至ることを初めて明らかにした。また、太陽光下での蜜蜂の農薬摂取を想定して、これらの分解特性を調べた。NMRスペクトル解析により、熱的には両農薬とも安定であり、紫外線に対してはジノテフランは安定であるもののクロチアニジンは不安定であることが判った。

《**キーワード**》ジノテフラン、クロチアニジン、ネオニコチノイド系農薬、蜂群、崩壊