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A clear difference in the impact on honeybee (*Apis mellifera*) colony between the two vehicles of sugar syrup and pollen paste

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ABSTRACT

Our previous study was carried out to examine the impact of honeybee (*Apis mellifera)* colony in which a neonicotinoid was administered using both sugar syrup and pollen paste as vehicles in our long-term field experiments. However, the effect of each vehicle (food) administered on the pesticide of a honeybee colony has not been investigated. This study investigates the difference in the impact of the neonicotinoid pesticide dinotefuran on honeybee (*A. mellifera*) colony between the two vehicles of sugar syrup and pollen paste. A distinct difference was observed between the two vehicles: The per-bee intake of dinotefuran administered through pollen paste as a vehicle until colony extinction was roughly one-fifth of the per-bee intake administered through sugar syrup, independently of dinotefuran concentrations. This difference can be attributed to the dissimilarity in strength of the impact on honeybee colony between worker bees which preferentially take sugar syrup (honey) to pollen paste and a queen bee and brood (larvae) which take pollen paste (bee bread) in preference to sugar syrup as a result of the long-lasting toxicity of dinotefuran. This suggests that pollen as a protein source contaminated by neonicotinoid pesticides can cause deeper adverse effect on a honeybee colony than honey as an energy source. A honeybee colony to which dinotefuran was administered becomes extinct after showing the appearance of a colony collapse disorder, as in the case of a colony administered with dinotefuran through both vehicles as reported in our previous study. It is inferred that a long-persistent pesticide such as neonicotinoid causes an overwintering failure due to the existence of toxicity of the pesticide in honey and bee bread stored in a hive which last even during overwintering.

Key words: Honey bee, sugar syrup, pollen paste, dinotefuran, pesticide, neonicotinoid, field experiment, long-term, colony, CCD, failure in wintering, collapse.

INTRODUCTION

Neonicotinoids, invented in the mid-1980s, have been commercially available since early 1990s, and their advantages and disadvantages have been extensively discussed. Serious threats posed to non-target animals, including human beings, have been reported. For example, it is strongly suspected that neonicotinoids have caused a significant worldwide decrease in freshwater arthropods, honeybees (*Apis mellifera*), butterflies, red dragonflies, and sparrows and have exerted adverse effects on the human brain through their neurotoxicity (Beketov and Liess, 2008; JEPA, 2010; Kimura-Kuroda et al., 2012a).

The decline in honeybee colonies has been investigated. As recorded in 2010–2011, around 30% winter losses of managed honeybee colonies in USA had occurred over 5 consecutive years (Van Engelsdorp et al., 2012). Neumann and Carreck (2010) reviewed data on honeybee colony losses and thereafter, proposed various causal theories. In USA, pesticides applied to crops, pesticides used in

apiculture and pesticide residues in hive products were reviewed and examined to determine what could play a role in colony collapse disorder (CCD) and other colony problems (Johnson et al., 2010). An extensive survey of honeybee colony losses was conducted in Japan in 2008 to 2010, which showed that bee loss was mostly due to neonicotinoids sprayed for stinkbug control following rice flowering (Taniguchi et al., 2012). A trend analysis of US agriculture demonstrated that a diminution in managed or wild pollinator populations could seriously threaten crop production (Calderone, 2012).

Systemic pesticides, of which neonicotinoids are representative, have direct, sublethal and indirect effects on species populations and ecosystems. They pose a new challenge to the ecological risk assessment of agrochemicals. The current risk protocol (LD_{50}), based on acute toxic effects, has been investigated. Laboratory testing of neonicotinoids (thiamethoxam, clothianidin, acetamiprid, and thiacloprid) demonstrated higher honeybee mortality than untreated controls (Laurino et al., 2011).

Nevertheless, LD_{50} was found inadequate in the absence of chronic exposure and cumulative, delayed impacts of new systemic compounds (Sánchez-Bayo et al., 2013). Under circumstances of the risk protocol of new agrochemicals such as neonicotinoids being yet to be established, many researches were conducted on the sublethal effects of neonicotinoids on bees. Pseudo-field testing showed that the foraging activity of honeybees decreased with pesticide concentrations of a few micrograms/kilograms (imidacloprid, fipronil) (Colin et al., 2004), and sublethal doses of imidacloprid were shown to affect the foraging behavior of honeybees (Yang et al., 2008). Furthermore, the effects of acute sublethal doses of acetamiprid and thiamethoxam on the behavior of honeybees under laboratory conditions and a particular vulnerability of honeybee behavior to sublethal doses of acetamiprid have been suggested (El Hassani et al., 2008). Under field conditions, a difference was observed in the survival rate between a group of bees treated with 70 ng imidacloprid and a control group (Visser and Blacquière, 2010). Colonies of bumblebees treated with field-realistic levels of imidacloprid showed significantly reduced growth rate and a production rate of new queens of about 15% of the control colonies (Whitehorn et al., 2012). Sublethal exposure of honeybees to thiamethoxam at levels that could put a colony at the risk of collapse caused high mortality due to homing failure (Henry et al., 2012). As a result, Matsumoto (2013) demonstrated that neonicotinoid and pyrethroid exposure reduced successful homing flights at doses far below the LD_{50} in the field where neonicotinoid caused reductions at relatively lower exposure than pyrethroid. Chronic exposure of bumblebees to imidacloprid at approximate field-level concentrations reduced the amount of pollen collected because of impaired foraging efficiency despite higher recruitment of workers for foraging and an increased number of workers lost outside. Such pollen constraints, coupled with fewer brood caretakers, resulted in reduced worker production, which exacerbated the problem of a reduced colony workforce. Imidacloprid works synergistically with λ-cyhalothrin, with increasing effects on the downward spiral of worker numbers (Gill et al., 2012). Sublethal oral doses of imidacloprid decreased the fecundity of worker bumblebees of queenless microcolonies, showing a dose dependence that principally correlated with nutrient limitations imposed by antifeedant effects (Laycock et al., 2012). Investigation of honeybee foraging behavior using the radiofrequency identification method showed that sublethal oral administration of clothianidin and imidacloprid impacted the flight frequency and duration of flight activity (Schneider et al., 2012). Honeybee behavior influenced by a sublethal oral dose of imidacroprid and acaricide were effectively measured using the video-tracking method (Teeters et al., 2012). Although a sublethal dose of imidacloprid had no effect on capped brood, pupation, and eclosion rates of honeybee larvae, the proboscis extension reflex test after emergence showed impairment of the development of olfactory ability (Yang et al., 2012). Assessment of the effects of imidacloprid ingestion by stingless bee larvae on their survival, development, neuromorphology, and adult walking behavior showed that these larvae were particularly susceptible to imidacloprid because the pesticide caused both high mortality and sublethal effects that impaired brain development and compromised mobility at the young adult stage (Van Tomé et al., 2012). In 2013, scientists from the European Food Safety Authority identified a number of risks posed by clothianidin (EFSA, 2013a), thiamethoxam (EFSA, 2013b), and imidacloprid (EFSA, 2013c) administered either as seed treatment or granules, with regard to their acute, chronic, and sublethal dose effects on colonies, larvae and behavior of bees.

Because the recommended concentrations of neonicotinoids, which are far more toxic than pesticides formerly used, are very low, they were not easily detectable. Moreover, the concentrations of pesticide residues in the environment have been reported. The active compound imidacloprid was detected in flowers, pollen, leaves, and corn in most field samples of maize crops treated with Gaucho seed dressing (Bonmatin et al., 2005). The concentration of neonicotinoids in guttation drops collected from plants seed-coated with neonicotinoids can be similar to or even higher than that of active ingredients commonly applied in field sprays for pest control (Girolami et al., 2009). The sudden death phenomenon of bees during sowing suggests the synergistic effect of high humidity and toxicity of a powder containing neonicotinoids (Marzaro et al., 2011). Samples of soil, plants, stored pollen in the hive, and dead bees near the hive entrance were contaminated by the neonicotinoids clothianidin and thiamethoxam (Krupke et al., 2012). Fifteen years of research on the risks

of using neonicotinoids at field concentrations for bee populations (honeybees, bumblebees, and solitary bees) was summarized while focusing on environmental residue levels, sublethal effects, and applicability for the evaluation of neonicotinoids in a pre-existing risk assessment scheme for systemic compounds (Blacquière et al., 2012).

Extensive supporting evidence suggests that neonicotinoids cause CCD, as earlier mentioned, while the risk protocol of neonicotinoids remains to be established. Neonicotinoids are a class of systemic pesticides that are absorbed by plants when applied to the soil, seeds, or leaves, circulating through the plants' tissues and killing the insects that feed on them. There are very few studies regarding the effect of neonicotinoids on honeybee colonies in Japan, although neonicotinoids have been used at much higher concentrations in Japan than in Europe or USA, with a higher concentration per unit area than that in most other countries. The present study tried to directly verify the neonicotinoid theory, which is a convincing theory based on experiments in which CCD was reproduced by field testing (Yamada et al., 2012). Another study that reported the influence of neonicotinoids on honeybee colonies through field experiments with apiaries was published in 2012 (Lu et al., 2012), around the same time frame as our previous study (Yamada et al., 2012).

In the present study, we report the results of the experiment which was conducted between July, 2011 and April, 2012, focusing on a difference in the influence of neonicotinoid pesticide on a honeybee (*A. mellifera*) between two vehicles of sugar syrup and pollen paste to administer the pesticide. Dinotefuran, which is the most commonly used neonicotinoid in Japan, was used in this study. Experimental concentrations of dinotefuran were determined based on the findings that clothianidin of 5 ppm was detected near a paddy field after aerial spraying (Kakuta et al., 2011) and the insecticidal activity of clothianidin to honeybees was roughly two and the half times as much as that of dinotefuran (Yamada et al., 2012). To clarify the difference between the long-term effects of a neonicotinoid pesticide ingested by honeybees through sugar syrup and pollen paste in this study, the neonicotinoid pesticide dinotefuran was administered through either sugar syrup or pollen paste at two concentrations each in a long-term field experiment, on the assumption that sugar syrup corresponds to honey and water in the natural environment and pollen paste corresponds to bee bread.

MATERIALS AND METHODS

Preparation of pesticide concentrations

The pathways where honeybees obtain pesticides from nectar, pollen, water and so on into a colony are very complicated. For examples, nectar and pollen contaminated with neonicotinoid pesticides are imported from fields into a hive, most of which are stored on combs as honey or bee bread after the pesticides are diluted with pesticide-free one and have an enduring effect on a honeybee-colony. Toxic water is fed to young bees and brood in the early spring or is used to reduce the temperature of cells in summer. This result is in contamination of the whole hive with the pesticides. Field experiments include many uncontrollable factors contrary to laboratory ones. To decrease ambiguity as much as possible, we tried to avoid unintentional contamination by pesticides. First, we selected an experimental apiary site where there were no large paddy fields and orchards in the vicinity where aerial-spraying are supposed to be conducted, whose pesticide-concentration is about 100 times as high as hand-spraying (for example, the concentration of dinotefuran is 12500 ppm in aerial-spray solution in Japan and 1000 ppm in hand-spray solution for extermination of stinkbugs). Secondly, we located a honeybee-watering place in the experimental apiary, where leaf mustard *Brassica juncea* and hairy vetch *Vicia villosa* were planted without pesticides to supply experimental honeybee colonies with pesticide-free water, nectar and pollen and to minimize the effects of environmental factors (Figures 1 and 2).

A field experiment was performed from 9th July, 2011 to 2nd April, 2012 under experimental conditions as shown in Table 1. STARKLE MATE ® (10% dinotefuran; Mitsui Chemicals Aglo, Inc., Tokyo, Japan) which is a commercial product and mostly sprayed on rice paddies in Japan was used in this study instead of dinotefuran only. This is to bring the experimental conditions closer to the realistic field ones. STARKLE MATE® includes auxiliary materials such as stabilizers, surfactants and adjuvants which are assumed to be biologically inert. Though the auxiliary materials may slightly affect honeybee colonies (Ciarlo et al., 2012), the present study expressed the experimental concentration of the active ingredient of the pesticide by the concentration of dinotefuran. Based on a concentration of 5 ppm clothianidin detected near a paddy field in which the pesticide was crop-dusted (Kakuta et al., 2011) and maximum residue limits (MRLs) of agricultural chemicals in foods in Japan (JFCRF, 2013) where the MRLs of dinotefuran ranged from 0.1 to 25 ppm, those of clothianidin 0.02 to 50 ppm and those of imidacloprid 0.05 to 10 ppm, the highest concentration of dinotefuran administered to a colony was determined to be 10 ppm. This is because the insecticidal activity of dinotefuran for stink bugs is about 0.4 times higher than that of clothianidin in a study by Yamada et al. (2012) and 5 ppm of clothianidin is equivalent to 12.5 ppm of dinotefuran. The concentrations of dinotefuran used in this study were 1 and 10 ppm (termed low and high, respectively) in sugar syrup and 0.565 and 5.65 ppm (termed low and high, respectively) in pollen paste. This is because pollen paste comprises pure pollen without dinotefuran and sugar syrup with dinotefuran in the ratio

Figure 1: Watering place in our apiary.

Figure 2: Flowering in our apiary.

1:1.3. Then a honeybee colony was termed where sugar syrup with 1 ppm of dinotefuran (DF) was fed, where pollen paste with 0.565 ppm of dinotefuran was fed, sugar syrup with 10 ppm of dinotefuran was fed and pollen paste with 5.65 ppm of dinotefuran was fed in "DF-Low/Syrup", "DF-Low/Pollen", "DF-High/Syrup" and "DF-High/Pollen",

Table 1: Details of experimental methods in this work.

Items	Details of experimental methods
Experimental period	July 9th 2011 to April 2nd 2012
Circumstances in an apiary	Without the large paddy field within a radius of two kilometers of the apiary and controllable time and place for crop-dusting
Confirmation of experiment	Double-checked by two persons
Number of hives	Five hives (one control and four dose tests)
Initial composition of a hive	Three combs and a feeder
Initial number of honeybees	ca. 3,000
Record of colony conditions	Photos of all combs and the inside of a hive with honeybees and all combs without honeybees taken in every experiment
Record of entrance circumstance	Time-lapse photography at the interval of 1 hour with two cameras
Number of adult bees in a hive	Directly counted with a counter on photos of all combs and the inside of a hive one by one
Number of brood in a hive	Directly counted with a counter on photos of all combs without honeybees
Number of dead bees	Directly counted one by one with distinguishing between dead bees in a hive and those on the tray installed under a hive
Intake of pesticide of honeybees	Accurately weighed by a weighing instrument
A kind of pesticide	Dinotefuran (DF) which is the top amount of pesticide used in Japan
Concentration of pesticide administered into a colony via sugar syrup or pollen paste	1 ppm (DF-Low) and 10 ppm (DF-High) of DF in sugar syrup. As pollen paste is made of sugar syrup and pure pollen in a ratio of 1.3:1 after pulverizing pollen into finer particles, the concentration of pesticide in pollen paste becomes 0.565 ppm for DF-Low/Pollen and 5.65 ppm for DF-High/Pollen. Sugar syrup was made of an equal amount of sugar and water.
Administration method of pesticide	1) Separate administration of either sugar syrup or pollen paste with pesticide in each hive; 2) For DF-High, the pesticide was administered only in the beginning of experiment (that is, the period of administration was seven days) and for the others, it continued to be administered during the experimental period till the colony become extinct
Prevention of swarming	Experiment start after the swarming period and then addition of a new comb and change to a bigger hive
Confirmation of a queen bee	Record by photos
Flowering site	Flowering site was installed in the apiary such as the fields of leaf mustard *Brassica juncea* and hairy vetch *Vicia villosa*.
Honeybee watering place	New watering place was provided for honeybees in the apiary
Hornet catcher	Installation of a hornet catcher in each hive after summer
Starting time of each experiment	Early morning if possible
Others	Record by photos about troubles such as wax worms, bee-beetles, etc

respectively. A concentration of dinotefuran in pollen paste was calculated from the relationship between ten parts of pollen without dinotefuran and thirteen parts of sugar syrup with 1 ppm of dinotefuran or 10 ppm.

Methods used in a field experiment

To enhance the accuracy of the experiment, it is necessary to increase the number of experimental colonies from the statistical point of view and to improve the accuracy of measurement in each experiment. In this study, we tried to improve the accuracy of measurement as thus explained below.

The numbers of honeybees and capped brood in a hive are often estimated from the weight of a hive and the area ratio on each comb occupied by sealed cells, respectively. These are a simple and easy method that can roughly grasp the change of the number of each member in a honeybee colony but may lack accuracy. For example, in the estimation of honeybee number using the weight method, the weight of an individual honeybee is incomparably lighter than the whole weight of a hive and the instrumental errors in measurement can be equivalent to the weight of a few hundred honeybees. Moreover, it is cumbersome to differentiate the

Figure 3: Experimental hives.

weight of honeybees from others such as capped brood honey, bee-bread, newly-piled-up comb and propolis etc in a hive which change with time. The estimation of the number of capped brood using the area method may include the difficulty when the area is occupied heterogeneously by dotted capped brood and the others. The present study tried to accurately count honeybees and capped brood one by one on the photos of combs and the inside of a hive. Though this study tried to develop a new automatic counting software system with the operation of binarizing photo images of combs and the inside of a hive, we cannot always succeed in their accurate counting was not successful because the exposure conditions to take photos are unstable in the field. The system cannot always accurately count overlaid bees, bees on a blurred image, bees on a low or an uneven contrast image and/or bees on a low brightness image, and capped brood differentiating from sealed honey in sealed cells even when the threshold is changed. Therefore, we tried to re-count honeybees and capped brood directly and patiently one by one with a hand-operated counter judging visually to aim for accuracy after they were roughly counted with the automatic counting software system.

Five hives, in each of which four combs (frames) numbered and ordered numerically and a feeder were installed, were sited on a hill facing east and being aligned north–south (Figure 3). When facing the front of a hive, the left side of the comb which was put into a hive was named "F "and the right side named "B". Four walls of a hive were numbered in the clockwise direction when viewed from the front of the hive and the bottom of the hive was named "B". These combs were always put in numerical order. The total number of adult bees on all combs and a feeder and the inside of a hive box (4 walls and the bottom) was counted directly from their photographs (sometimes enlarged) with a counter. The total number of capped brood was counted in a similar manner, after shaking the bees off each comb. The total number of dead bees in and around a hive was counted which was placed on a larger tray than a hive, one by one with a pair of tweezers. Consumption of foods (sugar syrup and pollen paste) by the honeybees was accurately measured with a weighing instrument after removing dead bees. A queen bee in a hive was photographically recorded on each observation date, similar to specific situations such as the presence of chalk brood or wax moth larvae and Asian giant hornet attacks. During the experimental period, the state of a hive was recorded with a digital camera, which was set in front of each hive, at one-hour intervals.

Table 2: Maximum Residue Limits of pesticides in Food in Japan updated on March 15th, 2013.

Pesticide	Maximum Residue Limits (MRLs) of Agricultural Chemicals in Foods in Japan updated on March 15th, 2013 [ppm]												Experimental concentration (ppm)(Beketov and Liess, 2008)
	Rice	Tea	Komatsuna	Spinach	Cabbage	Lettuce	Shungiku	Chinese cabbage	Soybeans	Corn	Wheat	Coffee	
Acetamiprid		30	5	3	3	5	5	0.5		0.2			
Imidacloprid	1	10	5	15	0.5	3	3	0.5	3	0.05	0.05	0.7	
Clothianidin	0.7	50	1	3	0.7	20	0.2	0.3	0.1	0.02	0.02	0.05	0.4, 0.8, 4
Dinotefuran	2	25	10	15	2	25	20	2	0.1				1, 2, 10
Thiamethoxan		15	5	10	2	3	3	2	0.02	0.02	0.02	0.2	
Nitenpyran	0.5	10	5	5	0.03	5	5	0.03	0.03	0.03	0.03	0.03	
Fipronil	0.01	0.002	0.002	0.002	0.05	0.002	0.002	0.1	0.002	0.02	0.002	0.002	
Fenitrothion	0.2	0.2	0.5	0.2	0.5	0.2	0.2	0.5	0.2	1	10		

1) It has been shown in previous work (Neumann and Carreck, 2010) that the concentrations of 4 ppm-clothianidin in bee-food and10 ppm-dinotefuran cause instant death of honeybees and those below 0.8 ppm of clothianidin and 2 ppm of dinotefuran cause gradual extinction of a colony.

A field experiment started in July 9th, after the swarming season, and was performed early in the morning on fine or cloudy days, before the foraging bees left a hive. They were performed in an apiary where honeybees could freely visit flowers in the field, thus allowing to avoid consumption of the food provided (sugar syrup and pollen paste containing a pesticide) in the case that the pesticide was repellent to honeybees. Both new foods (sugar syrup and pollen paste), which were prepared according to experimental conditions, were fed to a hive after weighing old ones that were removed from the hive every observation date. In DF-Low/Syrup and DF-Low/Pollen, the neonicotinoid pesticide, dinotefuran, continued to be administered into a honeybee colony till extinction or just before wintering in the case that the colony did not become extinct before wintering, respectively. On the other hand, in DF-High/Syrup and DF-High/Pollen, the pesticide was administered only for the first time around (for the first 7 days). The surviving colonies escaped from a collapse appeared vigorous before wintering. Experiments were recorded by still and moving photography, and the results were accessed at any time, if necessary.

RESULTS

Long-term observations

In this study, we will report the results of the 2011 experiment conducted between July in 2011 and April in 2012 while comparing them briefly with those of Yamada et al. (2012). Table 2 shows the observation results (the numbers of combs, queen, dead bees, mites and wax-moth larvae) of this study. Only dinotefuran was used in a colony in the 2011 experiment, being administered in the form of either sugar syrup or pollen paste, whereas both dinotefuran and clothianidin were used in 2010 in both vehicles. General information from these observations can give an overview of changes in experimental situations and conditions, such as the occurrence of CCD, incidences of chalk brood or wax moth larvae, and attack by Asian giant hornets. The number of dead bees is recorded qualitatively and quantitatively in Table 3.

Observational results of both years (previous and this works) showed that adult bees suffered almost instant death from high-concentration pesticides. No wax moth larva were present in the hive even after very few adults remained, and the repellent effect of neonicotinoids on honeybees seems to be weak, on the basis of the intake of colorless, tasteless, and odorless pesticides. A queen bee continued to survive to extinction of the colony accompanied by a sharp decrease in the number of adult bees and capped brood just after the start of the experiment. Thereafter, there was a further gradual decrease in the number of colonies with the appearance of CCD (the presence of a queen, some worker bees and brood, a stock of food, and few dead bees) and

Table 3: Summary of observations (numbers of combs, queen, dead bees, mites and wax-moth larvae).

Date	Days	Control					DF-Low/Syrup					DF-Low/Pollen					DF-High/Syrup					DF-High/Pollen				
		Combs	Queen	Dead bees	Mites	Wax- moth larvae	Combs	Queen	Dead bees[1)]	Mites	Wax- moth larvae	Combs	Queen	Dead bees	Mites	Wax- moth larvae	Combs	Queen	Dead bees	Mites	Wax- moth larvae	Combs	Queen	Dead bees	Mites	Wax- moth larvae
9-Jul-11	0	3	1	0	0	0	3	1	0	0	0	3	1	0	0	0	3	1	0	0	0	3	1	0	0	0
10-Jul-11	0	3	1	0			3	1	0			3	1	0			3	1	Many			3	1	Many		
16-Jul-11	7	3	1	2	0	0	3	1	444	0	0	3	1	113	0	0	3	1	3314	Few	0	3	0	1899	0	0
																	Stop of dinotefuran administration					Stop of dinotefuran administration				
22-Jul-11	13	4	1	0	0	0	3	1	137	0	0	3	1	113	0	0	3	1	7	0	0	3	0	319	0	0
29-Jul-11	20	5	1	5	0	0	3	1	279	0	0	3	1	110	0	0	3	1	130	0	0	3	0	196	3	1
6-Aug-11	28	5	1	0	0	0	3	1	152	0	0	3	1	45	0	0	3	1	80	0	0	3	0	2	0	1
																								Colony extinction (stock of foods)		
12-Aug-11	34	6	1	4	0	0	3	1	157	0	0	3	1	112	0	0	3	1	88	0	0					
18-Aug-11	40	6	1	12	0	0	3	1	59	0	0	3	1	115	0	0	3	1	69	0	0					
26-Aug-11	48	6	1	13	0	0	3	1	160	0	0	3	1	116	0	0	3	1	10	0	0					
10-Sep-11	63	6	1	14	0	0	3	1	115	0	0	3	1	144	0	0	3	1	42	0	0					
17-Sep-11	70	6	1	23	0	0	3	1	15	0	0	3	1	112	0	0	3	1	134	0	0					
24-Sep-11	77	6	1	862)	0	0	3	1	7	0	0	3	1	115	0	0	3	1	583[2)]	0	0					
29-Sep-11	82	6	1	6	0	0	3	1	6	0	Few	3	1	69	0	0	3	1	48	0	0					
7-Oct-11	90	6	1	4	0	0	3	1	9	0	Few	3	1	92	0	0	3	1	13	0	0					
21-Oct-11	104	6	1	2	0	0	3	0	1	0	Many	3	1	98	0	0	3	1	5	0	0					
							Colony extinction (stock of foods)																			
30-Oct-11	113	6	1	12	0	0						3	1	15	0	0	3	1	3	0	0					
4-Nov-11	118	6	1	5	0	0						3	1	87	1	0	3	1	1	0	0					
18-Nov-11	132	6	1	35	0	0						3	1	47	0	0	3	0	24	0	0					
26-Nov-11	140	6	1	305[2)]	0	0						3	1	316[2)]	0	0	3	0	59	0	0					
3-Dec-11	148	6	1	113[2)]	0	0						3	1	119[2)]	0	0	3	0	22	0	0					
												Stop of dinotefuran administration					Colony extinction (stock of foods)									
17-Dec-11	162	6	1									3														
			Start of wintering										Start of wintering													
16-Feb-12	223	6	1									3	0													
			During wintering									Colony extinction (stock of foods)														
2-Apr-12	269	6	1		1	1																				
			Finish of wintering																							

1) Total number of dead bees at the observation date = Number of dead bees outside the hive (on the large tray which is placed under the hive box) + Number of dead bees inside the hive; 2) Attacks by Japanese giant hornets (There were evidences such as dead hornets in front of tB32+B10: A+B10:AB33); [2]: (Blacquière et al., 2012).

Table 4: The numbers of adult bees, capped brood and dead bees.

Date	Days	Control			DF-Low/Syrup			DF-Low/Pollen			DF-High/Syrup			DF-High/Pollen		
		Pesticide-free			1 ppm of dinotefuran			0.565 ppm of dinotefuran			10 ppm of dinotefuran			5.65 ppm of dinotefuran		
		Adult	Brood	Dead	Adult	Brood	Dead	Adult	Brood	Dead	Adult	Brood	Dead	Adult	Brood	Dead
9-Jul-11	0	3392	5819	0	2965	4263	0	2158	2556	0	3295	3880	0	1659	6093	0
16-Jul-11	7	6827	3644	2	4257	1287	444	3755	2377	113	1102	986	3314	[430]	[2865]	[1899]
22-Jul-11	13	6399	4692	0	5233	1655	137	3493	3207	113	1407	232	7	[330]	[1165]	[319]
29-Jul-11	20	7737	4861	5	3131	1034	279	4025	2743	110	1451	939	130	[17]	[790]	[196]
6-Aug-11	28	7893	7179	0	2393	1591	152	4372	3480	45	1384	2370	80	[1]	[729]	[2]
12-Aug-11	34	7675	8390	4	1762	2288	157	4513	3217	112	1752	3217	88			
18-Aug-11	40	8873	6125	12	2298	1137	59	4829	1933	115	2257	946	69			
26-Aug-11	48	9327	5797	13	1623	1757	160	4276	2099	116	2271	913	10			
10-Sep-11	63	9249	8803	14	1253	318	115	4620	1343	144	2096	545	42			
17-Sep-11	70	9762	8327	23	997	341	15	4241	1546	112	1760	707	134			
24-Sep-11	77	11252	7034	86	462	453	7	3862	2693	115	908	772	583			
29-Sep-11	82	10736	5810	6	342	230	6	3792	2445	69	996	505	48			
7-Oct-11	90	12015	6100	4	169	109	9	4232	2082	92	1076	12	13			
21-Oct-11	104	11253	4100	2	[0]	[45]	[1]	4225	1935	98	860	220	5			
30-Oct-11	113	10958	4300	12				4044	2105	15	895	147	3			
4-Nov-11	118	10654	3900	5				3675	2665	87	790	245	1			
18-Nov-11	132	11303	3430	35				4022	1720	47	[779]	[160]	[24]			
26-Nov-11	140	12390	1750	305				3806	1034	316	[630]	[48]	[59]			
3-Dec-11	148	12109	2038	113				2992	840	119	[393]	[21]	[22]			
17-Dec-11	162	11811	212					2433	157		[0]	[0]				
16-Feb-12	223	10514	0					[0]	[0]							
2-Apr-12	269	9622	1873													

The red figures denote a state that foods (sugar syrup, pollen paste) with a pesticide (dinotefuran) were fed into a colony and the black ones denote pesticide-free foods. Foods with a pesticide were changed to pesticide-free ones in the morning of measurement date: E.g., in DF-High/Syrup and DF-High/Pollen, the change was made in the morning of July 16th. The [bracketed figures] denote a state that the queen had been lost.

extinction. Table 3 shows that CCD probably occurs along the pathway to colony extinction following administration of a pesticide, as reported in our previous study in 2010 experiment (Yamada et al., 2012).

Measurement of the number of adult bees, capped brood and dead bees

This work of the 2011 experiment differed from the one of 2010 experiment (Yamada et al., 2012) in that pesticide administration in the former was either through sugar syrup or pollen paste, while both were used in the latter (Yamada et al., 2012). Table 4 shows the numbers of adult bees, capped brood, and dead bees in this study.

Figures 4 to 6 show changes in the number of adult bees, capped brood, and dead bees in 2011, respectively. Experimental colonies except for DF-High/Pollen (DF-Low/Syrup, DF-Low/Pollen, DF-High/Syrup) dwindled away to nothing through the aspects of CCD (the existence of the queen with some bees and brood, stock of foods, few dead bees around a hive), similarly to our previous study (Yamada et al., 2012) conducted in 2010. The photographic image of CCD is shown in Figure 7. On the other hand, in DF-High/Pollen, the queen got lost in the first administration of pesticide. Administration of pesticide led to decreases in adult bees and brood (Figures 4 and 5).

Table 4 and Figures 4 to 6 suggest the followings under the support of the records at intervals of 1 h with digital cameras in front of the hives:

In DF-Low/Syrup where sugar syrup containing 1 ppm of dinotefuran continued to be fed to a colony until extinction, adult bees and capped brood gradually decreased in number under the existence of the queen and few dead bees and finally the colony became extinct. In DF-Low/Pollen where pollen paste containing 0.565 ppm of dinotefuran continued to be fed to a colony until the morning of November 26th, 2011 (just before wintering) and

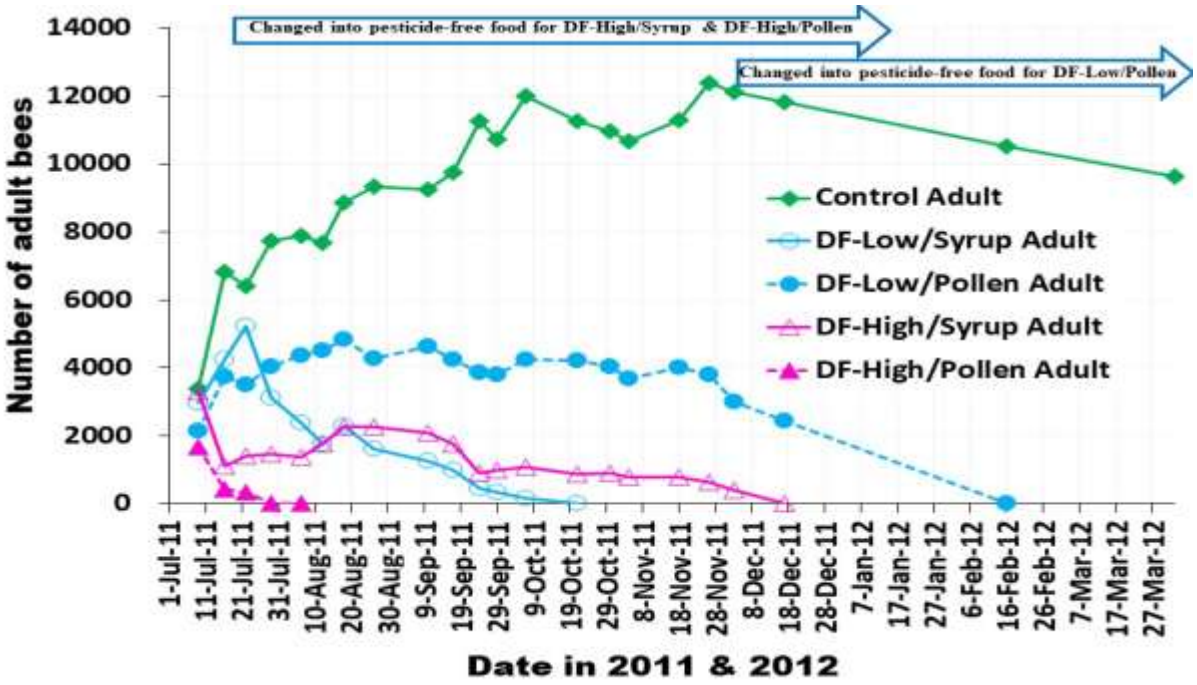

Figure 4: Change in number of adult bees. The pesticide (dinotefuran) was administered through sugar syrup or pollen paste, the latter consisting of 10 parts of pollen without dinotefuran and 13 parts of sugar syrup with dinotefuran. This was prepared by mixing the pollen substitute BB Food A® (Bee Culture Laboratory Co., Japan) with pure pollen at a ratio of 1:1. Control: without dinotefuran. DF-Low/Syrup: 1 ppm dinotefuran in sugar syrup. DF-Low/Pollen: 0.565 ppm dinotefuran in pollen paste, consisting of 10 parts of pollen without dinotefuran and 13 parts of sugar syrup with 1 ppm dinotefuran. DF-High/Syrup: 10 ppm dinotefuran in sugar syrup; the sugar syrup without dinotefuran was administered on and after July 16th, 2011. DF-High/Pollen: 5.65 ppm dinotefuran in pollen paste, consisting of 10 parts of pollen without dinotefuran and 13 parts of sugar syrup with 10 ppm dinotefuran; the pollen paste without dinotefuran was administered on and after July 16th, 2011. In DF-Low/Pollen, the administration of dinotefuran through pollen paste was discontinued with feeding pesticide-free sugar syrup on December 3rd, 2011. On December 17th, 2011, colonies of Control and DF-Low/Pollen entered their wintering after pesticide-free sugar syrup was discontinued. We observed the two colonies during wintering on February 16th, 2012 choosing a fine day in order to avoid an adverse effect on the colonies and found that the colony of DF-Low/Pollen had become extinct, whereas the colony of control survived.

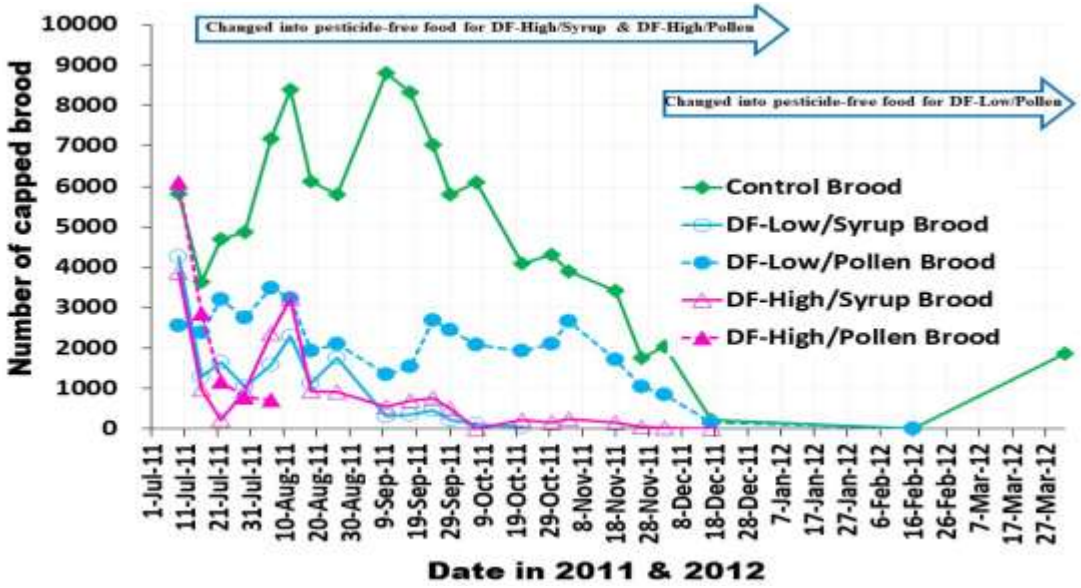

Figure 5: Change in number of capped brood.

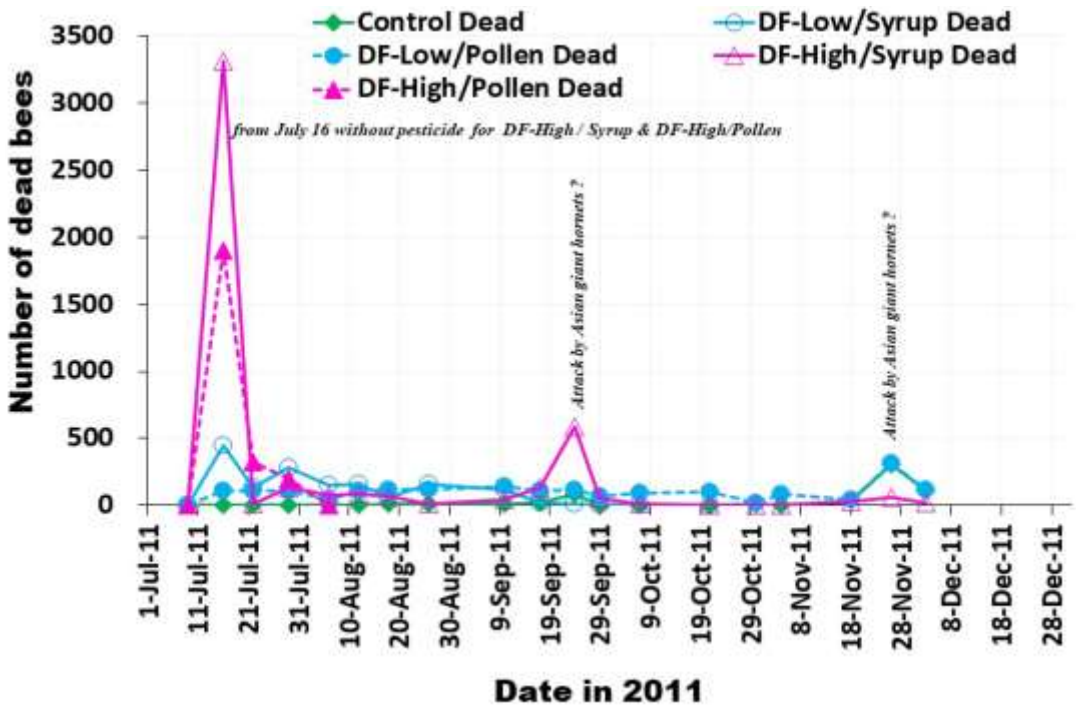

Figure 6: Change in number of dead bees. The marked increase in the number of dead bees observed immediately after the start of the experiment was due to pesticide (dinotefuran) toxicity. Incidentally, from mid-September, the sudden increase of dead bees was due to attack by Asian giant hornets.

Figure 7: Difference in state on comb at the start of experiment and just before colony extinction. The pesticide (dinotefuran) was administered into DF-Low/Syrup till the colony became extinct, but it was administered into DF-High/Syrup for seven days between the start of the experiment on July 9th and the next observation date July 16th. The queen remained alive on the verge of colony extinction in all colonies. The colony became extinction after giving the appearance of CCD as reported in our previous report (Yamada et al., 2012).

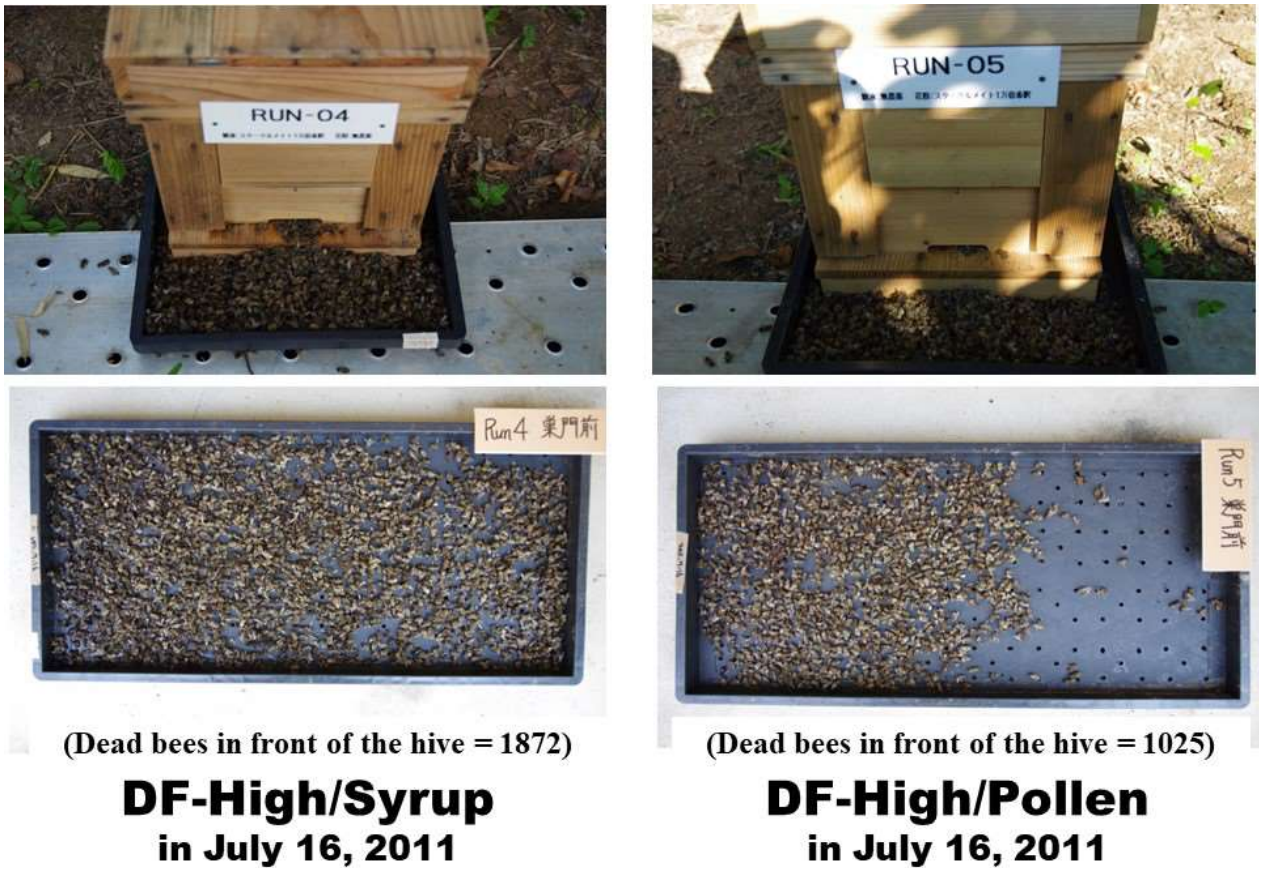

Figure 8: Dead bees in front of the hive in July 16th, 2011. The number of dead bees in front of the hive (on the tray) of DF-High/Syrup was 1, 872 while that of DF-High/Pollen was 1,025. The number of dead bees in the hive of DF-High/Syrup was 1, 442 and that of DF-High/Pollen 874. Therefore, the total number of dead bees of DF-High/Syrup was 3, 314 and that of DF-High/Pollen was 1, 899.

afterward dinotefuran-free one was fed until extinction, adult bees and capped brood did not much change in number until the start of wintering under the existence of the queen and few dead bees and they became zero during wintering. In DF-Low/Syrup and DF-Low/Pollen, the queen bee survived to colony extinction, with a gradual decrease in the number of adult bees and capped brood, and the presence of few dead bees giving the appearance of CCD prior to extinction.

In DF-High/Syrup where sugar syrup containing 10 ppm of dinotefuran was fed to a colony for first seven days (only once) and after that pesticide-free one continued to be fed, adult bees and brood sharply decreased in number with many dead bees immediately after the administration of pesticide as shown in Figure 8. Thus, they gradually decreased in number under the existence of the queen and few dead bees and finally became zero. In DF-High/Pollen where pollen paste containing 5.65 ppm of dinotefuran was fed to a colony for the first seven days (only once) and after that pesticide-free one continued to be fed, adult bees and brood sharply decreased in number with many dead bees without the queen immediately after the administration of pesticide as shown in Figure 8 and they decreased in number with some dead bees and finally the colony became extinct.

Colonies with DF-Low/Syrup, DF-High/Syrup, and DF-High/Pollen had already collapsed before wintering. Although the one surviving colony of DF-Low/Pollen appeared active just before wintering on December 3, with the number of adult bees just before wintering being greater than that at the start of the experiment on July 9th. Figure 9 shows the colony was confirmed to be unsuccessful in overwintering in mid-February of the following year. This is because the administration of pesticide through pollen mainly affected the brood and as a result, newly enclosing bees before wintering in DF-Low/Pollen became shorter in longevity than those in the control. The control colony succeeded in overwintering and was vigorous after the end of experiment (April 2nd, 2012).

In DF-High/Syrup and DF-High/Pollen, mass dead bees were found in front of a hive on the day after administration of the pesticide as described in Table 3 and as shown in Figure 8 of 7 days after. In DF-High/Syrup, the queen bee survived to colony extinction, with a sharp decrease in the number of adult bees and capped brood immediately after the administration of dinotefuran (Figure 10). This was

Figure 9: Comparison of the DF-Low/Pollen colony at the start of the experiment and just before wintering. The upper and lower images are representative combs with honeybees at the start of the experiment and immediately before wintering (existence of the queen bee confirmed), respectively. The colony appeared vigorous before wintering but became extinct during wintering.

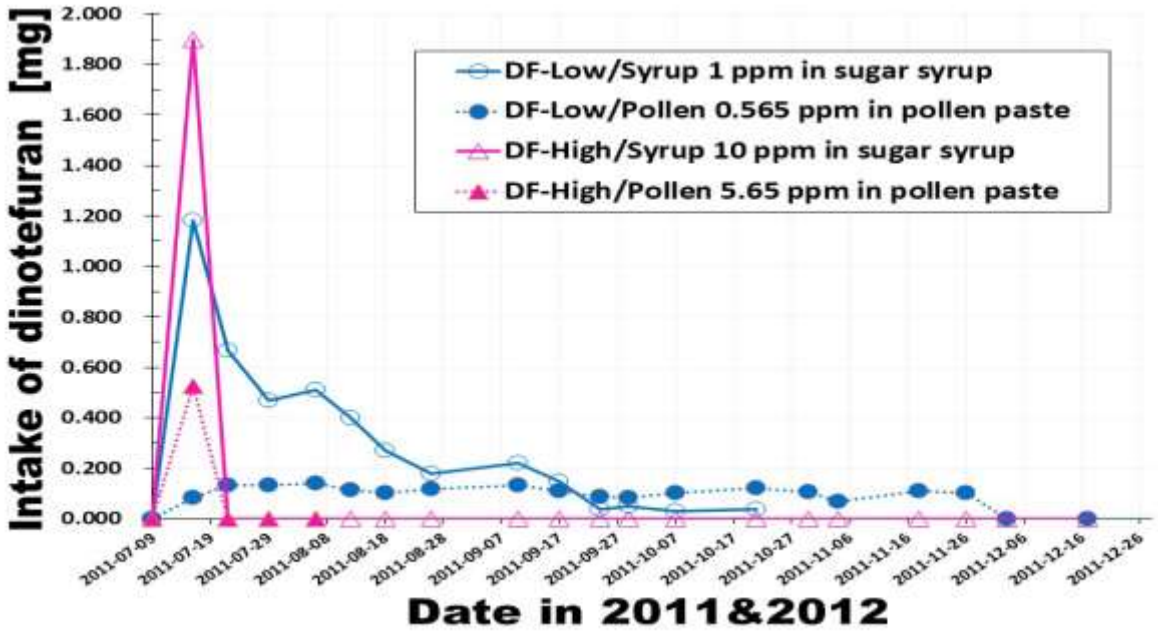

Figure 10: Change in the intake of dinotefuran. In DF-High/Sugar syrup and DF-High/Pollen, the pesticide (dinotefuran) was administered only once from the start of the experiment on July 9th in 2011 to the next observation date on July 16th (seven days). The intake of pesticide in DF-High/Pollen was greater than DF-Low/Pollen because the initial number of brood in DF-High/Pollen was more than twice that in DF-Low/Pollen. Intake of pesticide through sugar syrup (DF-Low/Syrup) appeared to depend on the number of adult bees and that through pollen paste (DF-Low/Pollen) depended on the number of brood. When a low-concentration pesticide is ingested through pollen (DF-Low/Pollen), the egg-laying capacity of the queen is less influenced by the pesticide as compared with newly emerged bees, which appear to be weak based on the high level of overwintering failure.

followed by a gradual decrease, with the colony giving the appearance of CCD where the queen and brood exist together with a small number of workers. Few dead bees were found near the hive and there were some bee-foods (honey, bee-bread) left. These results are similar to those of previous study (Yamada et al., 2012).

On the other hand, in DF-High/Pollen, the queen bee was lost immediately after the administration of dinotefuran, and there were many dead bees present. This was followed by a sharp decrease in the number of adult bees and capped brood and the colony rapidly became extinct. These results suggest that when a pesticide such as dinotefuran is administered through pollen paste, it impacts the queen bee more strongly than when it is administered through sugar syrup.

Figure 6 shows that the number of dead bees remained almost constant at a low level, except for the periods immediately after the administration of high-concentration dinotefuran and when attack by Asian giant hornets was recorded.

Cumulative total of the number of honeybees having taken pesticide in an experimental colony

To estimate the pesticide intake per bee, it is necessary to obtain a cumulative total of the number of honeybees (adult bees and brood) which have ingested the pesticide (dinotefuran) during the experiment. This is because the total amount of the pesticide ingested by the colony can be obtained from the amount of sugar syrup or pollen paste consumption.

Here, we consider two cases on a period when honeybees were exposed to a pesticide in an experimental colony: One is the shortest period case (CASE 1) where the pesticide was immediately ingested by honeybees soon after administration and the exposure period was approximately equal to the administration one; the other is the longest case (CASE 2) where the pesticide was partly stored in cells on a comb and continued to be ingested through the cells by honeybees until colony extinction even after the stop of pesticide administration. When the colony never becomes extinct, a cumulative total of the number of honeybees exposed to the pesticide will be liable to overestimated in CASE 2 because honeybees which ingest pesticide-free honey or bee bread stored in cells may be counted. CASE 1 is based on the assumption that the pesticide administered leads to the colony extinction while affecting honeybees (adult bees, brood etc.) only during the administration period of pesticide. CASE 2 is based on the assumption that it continues to affect all of honeybees in the colony till extinction even if it is discontinued halfway to be administered. The number of honeybees which seem to ingest the pesticide in CASE 1 is equal to that in CASE 2 when the experimental colony has become extinct before the administration of pesticide continued. In a control colony, the period to obtain the cumulative total of the number of honeybees is assumed to be the feeding period of food to the colony without making a distinction between CASE 1 and CASE 2.

The cumulative total of the number of honeybees having taken a pesticide in an experimental colony is defined by the sum of the numbers of initial adult bees, newborn ones for the duration of pesticide administration and capped brood at the final pesticide administration to the colony or at the colony extinction. The following assumptions were made for the estimation of the cumulative number of honeybees: (1) The age distribution of capped brood at an observation date is uniform between the first day when the cells of larvae are newly capped and the twelfth day when they eclose. (2) The number of adult bees that emerge from the pupae (capped brood) per day at a given day is one-twelfth of that of the capped brood at the last observation date before the day. (3) The total number of adult bees born between two successive observation dates is given by the product of one-twelfth of the number of capped brood at the former observation date and the number of days from the former to the latter observation date (4) The procedure in (3) is applied even when the number of days between two successive observation dates is greater than 12. (5) The number of capped brood at the time of the final pesticide administration or colony extinction is regarded as the number of adult bees having ingested the pesticide assuming that all the capped brood has already ingested the pesticide. (6) There is no newly capped brood after the middle of December because a queen stops laying eggs at the beginning of December in Japan.

Here, we give an example of the procedure for estimating the cumulative total of the number of honeybees shown in Table 4 for the DF-Low/Pollen colony from July 9th in 2011 to December 3rd when the pesticide administration into the colony was stopped (CASE 1) under the aforementioned assumptions. The number of initial adult bees is 2158; the cumulative total number of adult bees newborn between each two successive observation dates = (2556/12)(7-0) + (2377/12)(13-7) + (3207/12)(20-13) + (2743/12)(28-20) + (3480/12)(34-28) + (3217/12)(40-34) + (1933/12)(48-40) + (2099/12)(63-48) + (1343/12)(70-63) + (1546/12)(77-70) + (2693/12)(82-77) + (2445/12)(90-82) + (2082/12)(104-90) + (1935/12)(113-104) + (2105/12)(118-113) + (2665/12)(132-118) + (1720/12)(140-132) + (1034/12)(148-140) = 27781; and the number of brood at the stop of the pesticide administration (December 3rd) is 840. That is, the cumulative total number of honeybees for the DF-Low/Pollen colony in CASE 1 until the stop of the pesticide (dinotefuran) administration on December 3rd (during 148 days elapsed) is the sum (30779) of the number of initial bees (2158), the cumulative total of the number of newborn ones (27781) and the number of capped brood at the final observation (840). Similarly, the cumulative total

Table 5: Change in the intake of pesticide as an active ingredient in 2011 experiment.

Date	Elapsed days	Control				DF-Low/Syrup					
		Pesticide (dinotefuran) - free				1 ppm of dinotefuran in sugar syrup					
		Amount of consumption				Amount of consumption					
		Sugar syrup [g]		Pollen paste [g]		Sugar syrup [g]		Pollen paste [g]		Dinotefuran [mg]	
		Interval	Integrated	Interval	Integrated	Interval	Integrated	Interval	Integrated	Interval	Integrated
9-Jul-11	0	0	0	0	0	0	0	0	0	0.000	0.000
16-Jul-11	7	1500	1500	250	250	1180	1180	180	180	1.180	1.180
22-Jul-11	13	1500	3000	250	500	668	1848	165	345	0.668	1.848
29-Jul-11	20	1500	4500	250	750	470	2318	194	539	0.470	2.318
6-Aug-11	28	1500	6000	216	966	510	2828	206	745	0.510	2.828
12-Aug-11	34	1500	7500	215	1181	400	3228	144	889	0.400	3.228
18-Aug-11	40	1500	9000	224	1405	270	3498	122	1011	0.270	3.498
26-Aug-11	48	1500	10500	248	1653	180	3678	157	1168	0.180	3.678
10-Sep-11	63	1500	12000	250	1903	220	3898	116	1284	0.220	3.898
17-Sep-11	70	1500	13500	236	2139	150	4048	86	1370	0.150	4.048
24-Sep-11	77	1500	15000	226	2365	40	4088	48	1418	0.040	4.088
29-Sep-11	82	1500	16500	230	2595	50	4138	20	1438	0.050	4.138
7-Oct-11	90	1500	18000	211	2806	30	4168	11	1449	0.030	4.168
21-Oct-11	104	1500	19500	250	3056	[40]	[4208]	[10]	[1459]	[0.040]	[4.208]
30-Oct-11	113	1500	21000	250	3306						
4-Nov-11	118	1500	22500	219	3522						
18-Nov-11	132	1500	24000	240	3762						
26-Nov-11	140	1330	25330	239	4001						
3-Dec-11	148	1500	26830	242	4243						
17-Dec-11	162	0	26830	0	4243						
16-Feb-12	223	0	26830	0	4243						
2-Apr-12	269	0	26830	0	4243						

Date	Elapsed days	DF-Low/Pollen					
		0.565 ppm of dinotefuran in pollen paste					
		Amount of consumption					
		Sugar syrup [g]		Pollen paste [g]		Dinotefuran [mg]	
		Interval	Integrated	Interval	Integrated	Interval	Integrated
9-Jul-11	0	0	0	0	Integrated	0.0000	0.0000
16-Jul-11	7	1222	1222	146	0	0.0825	0.0825
22-Jul-11	13	1020	2242	239	146	0.1350	0.2175
29-Jul-11	20	1270	3512	239	385	0.1350	0.3525
6-Aug-11	28	1500	5012	247	624	0.1396	0.4921
12-Aug-11	34	810	5822	203	871	0.1147	0.6068
18-Aug-11	40	1000	6822	182	1074	0.1028	0.7096
26-Aug-11	48	1120	7942	212	1256	0.1198	0.8294
10-Sep-11	63	910	8852	235	1468	0.1328	0.9622
17-Sep-11	70	1090	9942	199	1703	0.1124	1.0746
24-Sep-11	77	470	10412	158	1902	0.0893	1.1639
29-Sep-11	82	360	10772	152	2060	0.0859	1.2498
7-Oct-11	90	610	11382	185	2212	0.1045	1.3543
21-Oct-11	104	1150	12532	219	2397	0.1237	1.4780
30-Oct-11	113	1500	14032	192	2616	0.1085	1.5865

Table 5: Conts. Change in the intake of pesticide as an active ingredient in 2011 experiment.

4-Nov-11	118	1120	15152	122	2808	0.0689	1.6554
18-Nov-11	132	1500	16652	196	2930	0.1107	1.7661
26-Nov-11	140	860	17512	181	3126	0.1023	1.8684
3-Dec-11	148	350	17862	123	3307	0.0000	1.8684
17-Dec-11	162	290	18152	0	3430	0.0000	1.8684
16-Feb-12	223	[0]	[18152]	[0]	3430	[0]	[1.8684]
2-Apr-12	269	0	0	0	[3430]	0.0000	0.0000

Date	Elapsed days	DF-High/Syrup					
		10 ppm of dinotefuran in sugar syrup					
		Amount of consumption					
		Sugar syrup [g]		Pollen paste [g]		Dinotefuran [mg]	
		Interval	Integrated	Interval	Integrated	Interval	Integrated
9-Jul-11	0	0	0	0	0	0.000	0.000
16-Jul-11	7	190	190	161	161	1.900	1.900
22-Jul-11	13	90	280	113	274	0.000	1.900
29-Jul-11	20	1010	1290	164	438	0.000	1.900
6-Aug-11	28	370	1660	158	596	0.000	1.900
12-Aug-11	34	220	1880	143	739	0.000	1.900
18-Aug-11	40	170	2050	105	844	0.000	1.900
26-Aug-11	48	220	2270	106	950	0.000	1.900
10-Sep-11	63	280	2550	114	1064	0.000	1.900
17-Sep-11	70	380	2930	82	1146	0.000	1.900
24-Sep-11	77	80	3010	69	1215	0.000	1.900
29-Sep-11	82	40	3050	30	1245	0.000	1.900
7-Oct-11	90	60	3110	46	1291	0.000	1.900
21-Oct-11	104	40	3150	70	1361	0.000	1.900
30-Oct-11	113	80	3230	33	1394	0.000	1.900
4-Nov-11	118	200	3430	20	1414	0.000	1.900
18-Nov-11	132	[170]	[3600]	[34]	[1448]	[0]	[1.900]
26-Nov-11	140	[50]	[3650]	[10]	[1458]	[0]	[1.900]
3-Dec-11	148	[10]	[3660]	[10]	[1468]	[0]	[1.900]
17-Dec-11	162	[0]	[3660]	[0]	[1468]	[0]	[1.900]
16-Feb-12	223						
2-Apr-12	269						

Date	Elapsed days	DF-High/Pollen					
		5.65 ppm of dinotefuran in pollen paste					
		Amount of consumption					
		Sugar syrup [g]		Pollen paste [g]		Dinotefuran [mg]	
		Interval	Integrated	Interval	Integrated	Interval	Integrated
9-Jul-11	0	0	0	0	0	0.000	0.000
16-Jul-11	7	[90]	[90]	[93]	[93	[0.525]	[0.525]
22-Jul-11	13	[220]	[310]	[87]	[180]	[0]	[0.525]
29-Jul-11	20	[820]	[1130]	[53]	[233]	[0]	[0.525]
6-Aug-11	28	[0]	[1130]	[0]	[233]	[0]	[0.525]
12-Aug-11	34						
18-Aug-11	40						

Table 5: Conts. Change in the intake of pesticide as an active ingredient in 2011 experiment.

26-Aug-11	48
10-Sep-11	63
17-Sep-11	70
24-Sep-11	77
29-Sep-11	82
7-Oct-11	90
21-Oct-11	104
30-Oct-11	113
4-Nov-11	118
18-Nov-11	132
26-Nov-11	140
3-Dec-11	148
17-Dec-11	162
16-Feb-12	223
2-Apr-12	269

number of honeybees for the DF-Low/Pollen colony in CASE 2 is the sum (31076) of the number of initial bees (2158), the cumulative total of the number of newborn ones (28918) obtained from the equation that 27781 + (840/12)(162-148) + 157 and the number of capped brood at final observation (0).

According to the aforementioned procedure, a cumulative total of the number of adult bees for each colony was estimated from capped brood and initial adult bees. Table 6 and Figure 11 show the estimated total number of adult bees.

Intake of foods (sugar syrup, pollen paste)

Table 7 shows the total intakes of foods (sugar syrup, pollen paste) and dinotefuran (active ingredient) taken from foods during experiment. It can be seen from Table 7 that the total intake of foods per bee seems to be independent of the dinotefuran (pesticide) content in food except for DF-High/Pollen whose colony became extinct by the little intake of toxic pollen paste containing 5.65 ppm dinotefuran; for sugar syrup, 0.3672 g/bee in Control, 0.3107 g/bee in DF-Low/Syrup, 0.5842 g/bee in DF-Low/Pollen, 0.5593 g/bee in DF-High/Syrup; for pollen paste, 0.0581 g/bee in Control, 0.1077 g/bee in DF-Low/Syrup, 0.1104 g/bee in DF-Low/Pollen, 0.2243 g/bee in DF-High/Syrup. This means that dinotefuran is hardly repellent to honey bees.

Total intake of pesticide per bee

The total intake of pesticide per bee is calculated by dividing the total intake of pesticide per colony by the cumulative total number of honeybees. Now, we give an example of the procedure for estimating the total intake of pesticide per bee for the DF-Low/Pollen colony in CASE 1: The total intake of the pesticide through pollen paste per colony is 1.8692 mg and the cumulative total of the number of honeybees is 30779 heads. Therefore, the total intake of pesticide per bee through pollen paste is $1.8692\times10^6/30779$ = 60.73 ng/bee in CASE 1 of DF-Low/Pollen. Similarly, that in CASE 2 is $1.8692\times10^6/31076$ = 60.15 ng/bee.

Table 7 shows the total intake of pesticide (dinotefuran) through sugar syrup per colony or that through pollen paste, the cumulative total number of honeybees in each colony obtained according to the procedure mentioned above, and the total intakes of dinotefuran by individual bees during a period of pesticide administration (CASE 1) or until extinction (CASE 2) through either sugar syrup or pollen paste in this work.

Here, we will discuss the total intake of pesticide per bee in the case (CASE 1) where the pesticide is ingested by the colony only during the administration period of pesticide. Figure 12 shows the total intake of dinotefuran per bee until colony extinction. Figure 12 and Table7 show that the total intake of dinotefuran per bee in DF-Low/Syrup (310.7 ng/bee) is about 5.1 times as much as that in DF-Low/Pollen (60.73 ng/bee), and that in DF-High/Syrup (290.3 ng/bee) is about 4.5 times as much as that in DF-High/Pollen (65.08 ng/bee). Also, total intake of dinotefuran per bee in DF-High/Syrup

Table 6: A cumulative total of the number of estimated adult bees estimated from capped brood

Date	Days	Control		DF-Low/Syrup		DF-Low/Pollen		DF-High/Syrup		DF-High/Pollen	
		Interval[1)]	Total[2)]	Interval	Total	Interval	Total	Interval	Total	Interval	Total
9-Jul-11	0	0	3392	0	2965	0	2158	0	3295	0	1659
16-Jul-11	7	3394	6786	2487	5452	1491	3649	2263	5558	3554	5213
22-Jul-11	13	1821	8607	644	6096	1189	4838	986	6544	2865	8078
29-Jul-11	20	2737	11344	965	7061	1871	6709				
6-Aug-11	28	3241	14585	689	7750	1829	8538				
12-Aug-11	34	3590	18175	796	8546	1740	10278				
18-Aug-11	40	4195	22370	1144	9690	1609	11887				
26-Aug-11	48	4083	26453	758	10448	1289	13176				
10-Sep-11	63	7246	33699	2196	12644	2624	15800				
17-Sep-11	70	5135	38834	186	12830	783	16583				
24-Sep-11	77	4857	43691	199	13029	902	17485				
29-Sep-11	82	2931	46622	189	13218	1122	18607				
7-Oct-11	90	3873	50495	153	13371	1630	20237				
21-Oct-11	104	7117	57612	127	13498	2429	22666				
30-Oct-11	113	3075	60687	45	13543	1451	24117				
4-Nov-11	118	1792	62479			877	24994				
18-Nov-11	132	4550	67029			3109	28103				
26-Nov-11	140	2287	69316			1147	29250				
3-Dec-11	148	1167	70483			689	29939				
17-Dec-11	162	2378	72861			980	30919				
16-Feb-12	223	212	73073			157	31076				

1) Interval: The number of adult bees which have newly emerged in each interval; 2) Total: A cumulative total of the number of adult bees; [1]: (Beketov and Liess, 2008); [2]: (Blacquière et al, 2012).

and DF-High/Pollen are almost the same as that in DF-Low/Syrup and DF-Low/Pollen, respectively.

DISCUSSION

Effect of dinotefuran (neonicotinoid pesticide) on adult bees and brood

Here, we discussed the effect of dinotefuran on the number of adult bees and brood on the basis of experimental results from 2011 (Table 4 and Figures 4 and 5).

High concentrations of dinotefuran, such as those in DF-High/Syrup and DF-High/Pollen, resulted in the presence of many dead bees near the hive and in the feeder within a few hours of administration. Considering that the concentration of pesticides sprayed on fields is about 10-fold higher than that in the experimental study of 2011, colonies can be presumed to collapse as follows: Foraging bees are instantly killed near the region where a high concentration of pesticide is sprayed directly because they consume water, nectar, or pollen containing pesticide. Instant death of foraging bees brings about a change in bee role, with house bees becoming foraging bees, thus, resulting in the lack of house bees and consequentially, the collapse of the colony. The number of adult bees decreases markedly immediately after the temporary and brief

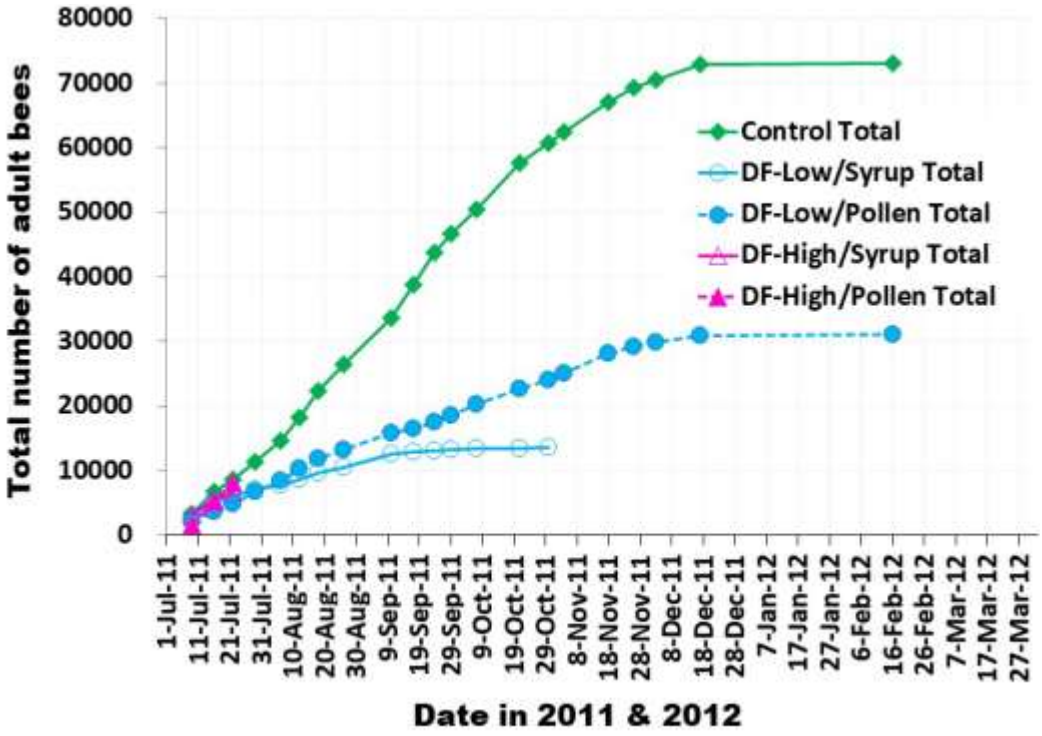

Figure 11: Cumulative total of the number of honeybees.

administration of a high-concentration pesticide. Even after discontinuation of administration, their numbers continue to decrease, leaving just a queen and a very small number of dead bees in and around the hive. The reason why foraging bees cannot return to the hive is the nervous disorders or debility due to the toxicity of the pesticide ingested by the capped brood before becoming adult bees. This means that the queen does not die but lays only few eggs because of reduced egg-laying capacity and an imbalanced colony. Short time administration of pesticides with high concentrations leads to the instant death of many honeybees with acute toxic symptoms. Yet, the subsequent pesticide-free administration finally leads to the colony extinction after presenting an appearance of a CCD because of the imbalanced colony structure and the reduced egg-laying performance of the queen etc.

Continuous administration of a low-concentration pesticide (about 1% of the concentration recommended to exterminate stink bugs) causes colony collapse, suggesting CCD, with the only major difference between the vehicles used being that colony collapse occurs earlier with sugar syrup than with pollen paste. This difference appears to be because of variation in total pesticide intake. First, intake from pollen paste is less than that from sugar syrup, and second, the intake period of pollen paste, which is mainly consumed by capped brood, is shorter than that of sugar syrup, which is mainly consumed by adult bees. This is because the period of capped brood is shorter than that of adults. This suggests that following pesticide administration, recently born adult bees live lesser than controls, despite the reduced load on them during wintering. In this regard, the results of this study substantially agree with those of previous study (Yamada et al., 2012).

The results of low-concentration pesticide administration suggest that foraging bees consuming water with pesticides diluted in a rice paddy are rarely killed on the sprayed site, return to the hive, and then transfer it to the colony members and the toxic water finally leads to colony collapse.

In other words, the pesticide in pollen paste (that is, bee bread) seems to act preferentially on capped brood and the queen as opposed to adult bees. As a result, at high concentrations, a lower intake from pollen paste collapses the colony earlier than that from sugar syrup (that is, honey). When a low-concentration pesticide is administered by dissolving in the water of a rice paddy or orchard after being sprayed, it will continue to affect the colony for a long time. Since the period of capped brood is very short, low-concentration pesticide has little effect on the capped brood but has an effect on the longevity of the queen, resulting in the inhibition of her egg-laying capacity. Finally, a colony is destined to collapse or fail in overwintering. Even if it does not collapse and appears active, pesticides impede egg laying of the queen and causes a decrease in the colony strength, while occasionally leading to infestation by mites, viruses, etc.

After ingesting water, nectar, or pollen with low-concentration pesticide, foraging bees carry the mildly toxic pesticides back to the hive. The toxicity in nectar is

Table 7: Intake of foods (sugar syrup, pollen paste) and pesticide (dinotefuran) in this work performed from July 9th in 2011 to April 2nd 2012.

Active ingredient (AI)	**Control**	**DF-Low/Syrup**	**DF-Low/Pollen**	**DF-High/Syrup**	**DF-High/Pollen**
	Pesticide-free	**Dinotefuran**	**Dinotefuran**	**Dinotefuran**	**Dinotefuran**
Vehicle (food) to administer a pesticide	nothing	Sugar syrup	Pollen paste	Sugar syrup	Pollen paste
Pesticide-free food fed into a colony	Sugar syrup and pollen paste	Pollen paste	Sugar syrup	Pollen paste	Sugar syrup
Feeding period of pesticide-free food	from July 9th to Dec 17	from July 9th to Oct 21	from July 9th to Dec. 3	from July 9th to Dec 17	from July 9th to Aug 6
Pesticide concentration in a vehicle[1)]	0 ppm of DF	1 ppm of DF	0.565 ppm of DF[2)]	10 ppm of DF	5.65 ppm of DF[2)]
Pesticide administration method		Continuous	Continuous	First one time	First one time
Pesticide administration period		from July 9th to Oct 21	from July 9 to Dec 3	from Jul 9 to Jul 16	from Jul 9 to Jul 16
Total intake of pesticide (dinotefuran) per colony	0.0000	4.2080	1.8692	1.9000	0.5257
CASE 1: Pesticide administration period to a experimental colony or food feeding period to a control colony					
Cumulative total of the number of honeybees per colony[3)]	73074	13543	30779	6544	8078
Period covered by the cumulative total of the number of honeybees per colony	from Jul 9 to Dec 17 in 2011	from Jul 9 to Oct 21 in 2011	from Jul 9 to Dec 3 in 2011	from Jul 9 to Jul 16 in 2011	from Jul 9 to Jul 16 in 2011
Total intake of pesticide per bee [AI ng/bee][5)]	0.00	310.70	60.73	290.30	65.08
Period covered by the total intake of pesticide per bee [AI ng/bee][5)]	from Jul 9 to Dec 17 in 2011	from Jul 9 to Oct 21 in 2011	from Jul 9 to Dec 3 in 2011	from Jul 9 to Jul 16 in 2011	from Jul 9 to Jul 16 in 2011
Total intake of sugar syrup per colony [g]	26830 (pesticide-free)	4208 (1 ppm of DF)	18152 (pesticide-free)	190 (10 ppm of DF)	90 (pesticide-free)
Total intake of pollen paste per colony [g]	4243 (pesticide-free)	1459 (pesticide-free)	3430 (0.565 ppm of DF)	161 (pesticide-free)	93 (5.65 ppm of DF)
Total intake of sugar syrup per bee [g/bee]	0.367	0.311	0.590	0.029	0.011
Total intake of pollen paste per bee [g/bee]	0.058	0.108	0.111	0.025	0.012
CASE 2 : Period from the start of experiment to the colony extinction or to a experimental colony or food feeding period to a control colony					
Cumulative total of the number of honeybees per colony[3)]	73074	13543	31076	13457	8582
Period of cumulative total of the number of honeybees per colony[3)]	from Jul 9 to Dec 17 in 2011	from Jul 9 to Oct 21 in 2011	from Jul 9 in 2011 to Feb 16 in 2012	from Jul 9 to Dec 17 in 2011	from Jul 9 to Aug 6 in 2011
Total intake of pesticide per bee [AI ng/bee][4)]	0.00	310.70	60.15	141.20	61.26
Total intake of pesticide per bee [AI ng/bee][4)]	from Jul 9 to Dec 17 in 2011	from Jul 9 to Oct 21 in 2011	from Jul 9 in 2011 to Feb 16 in 2012	from Jul 9 to Dec 17 in 2011	from Jul 9 to Aug 6 in 2011

Table 7: Intake of foods (sugar syrup, pollen paste) and pesticide (dinotefuran) in this work performed from July 9th in 2011 to April 2nd 2012.

Total intake of sugar syrup per colony [g]	26830 (pesticide-free)	4208 (1 ppm of DF)	18152 (pesticide-free)	3660 (190g with DF of 10 ppm and the rest without pesticide)	1130 (pesticide-free)
Total intake of pollen paste per colony [g]	4243 (pesticide-free)	1459 (pesticide-free)	3430 (0.565 ppm of DF)	1468 (pesticide-free)	233 (93g with DF of 5.65 ppm and the rest without pesticide)
Total intake of sugar syrup per bee [g/bee]	0.367	0.311	0.584	0.272	0.132
Total intake of pollen paste per bee [g/bee]	0.058	0.108	0.110	0.109	0.027

1) Dinotefuran concentration as an active ingredient (A.I.); 2) Pollen paste was made of 13 parts of sugar syrup with dinotefuran and 10 parts of pollen without pesticide in weight: E.g., as pollen paste in DF-Low/Pollen is composed of 13 parts of sugar syrup with 1 ppm dinotefuran and 10 parts of pollen without dinotefuran, the concentration of dinotefuran in pollen paste becomes 1 [ppm]x13/23≒0.565 [ppm]; 3) Estimated from the sum of the number of initial adult bees, the cumulative number of newly-born adult bees by eclosion of capped brood and the number of capped brood at the final observation; 4) Obtained by dividing the total intake of pesticide (dinotefuran) by the cumulative total number of honeybees: E.g., for DF-Low/Pollen, the total intake of pesticide per bee from July 9 to December 3 is given by 1.8692 [mg] / 30779 ≒ 60.73 [ng]/bee in the case of CASE 1, and is similarly given by 1.8692 [mg] / 31076 ≒ 60.15 [ng]/bee in the case of CASE 2; [1]: (Beketov and Liess, 2008); [3]: (Bonmatin et al., 2005); [4]: (Calderone, 2012).

diluted by new nectar from flowers. The mildly toxic pesticides are then ingested by house bees, capped brood, and the queen, or stored in combs as honey and bee bread. When capped broods that ingest the mildly toxic water and bee bread become foraging bees, they are unable to return to the hive because of either disorientation or exhaustion due to chronic toxicity. The egg-laying capacity of the queen declines through ingestion of mildly toxic pesticides, but she survives colony collapse. The death of many foraging bees creates an imbalance in the proportion of house bees, foraging bees, capped brood, and larvae in a colony, leading to colony collapse.

Difference in effects of pesticide intake through sugar syrup and pollen paste on colony

We now focus on the total intake of pesticide per bee to colony extinction in Table 7 and Figure 12 which show the integrated intake of food (sugar syrup or pollen paste) per colony and toxic food, food per bee during experiment, and food and an active ingredient (dinotefuran) during feeding on toxic food. The discussion about the impact of dinotefuran (pesticide) on a honeybee colony is thus described hereafter.

The reasons for the total intake of dinotefuran (active ingredient) per bee through sugar syrup appearing to be greater than its LD_{50} value (Iwasa et al., 2004; EPA, 2004) are as follows: (1) It comes from the difference between chronic toxicity and acute one. (2) A certain amount of dinotefuran is stored in combs as noxious honey and bee bread, whose quantity changes with weather and season. (3) The number of adult bees ingesting dinotefuran incorporates an estimation error. (4) Judging from the estimated amount of sugar taken by honeybees in the previous literature (Rortais et al., 2005) (about 1500 mg/bee at a maximum. from larvae to foraging bees), which equals half as much as the amount of sugar syrup consisting of even amounts of sugar and water, the individual intakes of sugar syrup in Table 7 (367.2 mg in Control, 310.7 mg in DF-Low/Syrup, 584.2 mg in DF-Low/Pollen, 559.3 mg in DF-High/Syrup, 139.9 mg in DF-High/Pollen) are less than 3000 mg/bee and seem not to be contradictory considering chronic toxicity.

On the other hand, the total intake of dinotefuran per bee through pollen paste is of the same order of magnitude as LD_{50}. Then, the individual intakes of pollen paste in Table 7 (58.1 mg in Control, 107.7 mg in DF-Low/Syrup, 110.4 mg in DF-Low/Pollen, 224.3 mg in DF-High/Syrup, 28.8 mg in DF-High/Pollen) seems to be reasonable judging from the weight of pollen consumed (112.5 mg to 195 mg) (reported by Crailsheim et al.,1992), which equals ten twenty-thirds times as much as the amount of pollen paste consisting of one pollen and 1.3 sugar syrup. This means that pollen paste is taken without much stock at the colony extinction. Table 7 and Figure 12 show that the total intake of dinotefuran per bee through sugar syrup to colony extinction is about five-fold (about 5.1-fold at a low concentration, about 4.5-fold at a high concentration) as much as that through pollen paste. The total intake of dinotefuran per bee until extinction through sugar syrup and that through pollen paste are almost constant respectively independent of its concentration.

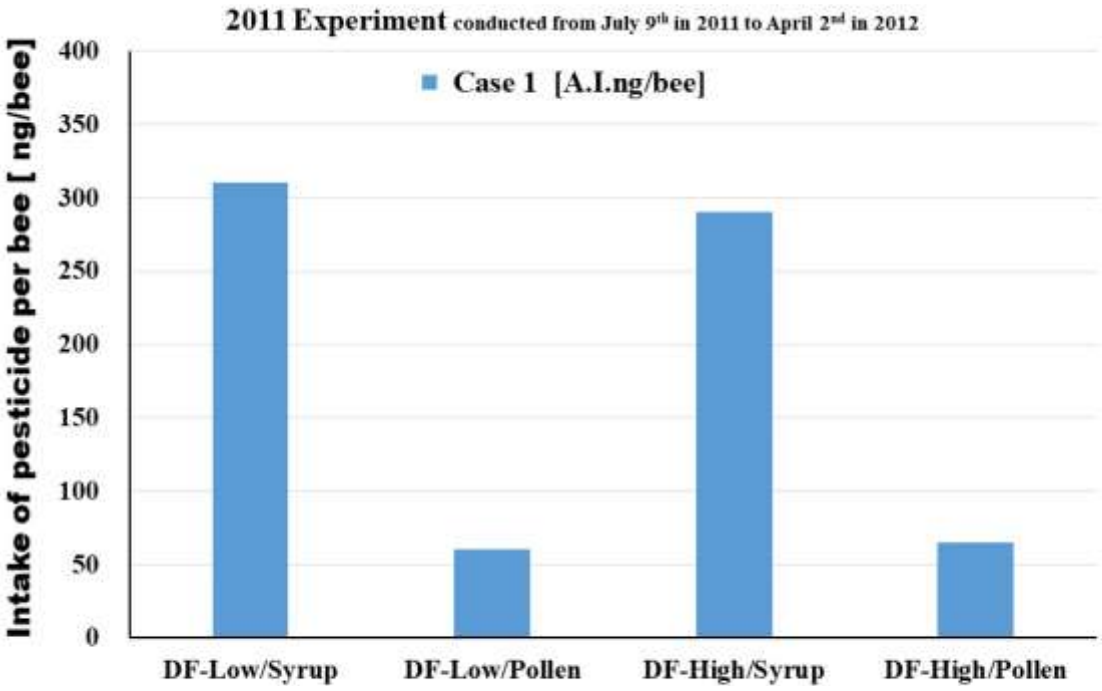

Figure 12: Total intake of dinotefuran per bee.

These results suggest that toxic pollen (pollen paste) containing neonicotinoids, such as dinotefuran, causes more terrible effects than toxic nectar or water (sugar syrup) because the total intake of dinotefuran through sugar syrup is almost five times as much as that through pollen paste. Also, they are probably poorly metabolized and mostly accumulate chronically in the body tissues of bees at low concentration because the effect of dinotefuran is independent of concentration as pointed out in previous study (Yamada et al., 2012). Since proteins are responsible for nearly every task of cellular life, toxic pollen contaminated by neonicotinoid pesticides (toxic proteins) may affect cell functions adversely over a long-term period of time because of their long- persistence. The damage inflicted by toxic pollen on cells and/or genes shortens the life-span of honeybee. We will discuss the difference in the impact of neonicotinoid, such as dinotefuran, on the longevity of a honeybee colony between the two vehicles of sugar syrup (honey) and pollen paste (bee bread) in the near future other than the toxic effectiveness which is reported in this study.

Conclusion

This study reconfirmed the findings of previous study (Yamada et al., 2012) that neonicotinoid such as dinotefuran results in the collapse and extinction of bee colonies after a honeybee colony assumed the appearance of CCD. The present result suggest that the insecticidal activity on a bee colony using pollen paste is roughly five times more than that using sugar syrup (honey or water), independent of pesticide concentration. When a pesticide is sprayed and dissolved in the water of a rice paddy or orchard at low concentration, the pesticide transported by foraging bees continues to affect the colony for a long time and finally leads to colony collapse or failure of overwintering. Even when a colony does not collapse and appears active, insecticidal toxicity impedes the queen's egg-laying capacity, weakens the colony and occasionally leads to mite infestation in the colony.

Pesticide intake per bee to colony extinction is greater than the LD_{50} of honeybees, suggesting that the overall longevity of a colony, which behaves as one living creature, cannot be assessed by LD_{50}, which is an indicator of individual's susceptibility to acute toxicity. Pesticides impact honeybee colonies both acutely and chronically. In addition to LD_{50} attributable to acute toxicity for individual bees, an indicator yielding information on colony changes is urgently needed in order to assess long-term pesticide effects on a complicated colony system exposed to toxicity ranging from chronic to acute.

Based on the aforementioned discussion and previous study (Yamada et al., 2012), a neonicotinoid of very low concentration, which cannot be analytically detected, continued to gradually accumulate in the tissues of organisms over a long term, causing great harm to them. Due to their high toxicity and relative non-degradability, neonicotinoids may be described as agro-poisons which can be persistent over a prolong period of time rather than agro-chemicals which can be degradable in a short period. Neonicotinoids may be poorly metabolized and are mostly

accumulated chronically in the tissues of bees at low concentrations. They may also pose a toxic threat to humans (JEPA, 2010; Taira, 2012a, b; Kimura-Kuroda et al., 2012b), and we are apprehensive of a nightmare scenario in which "Harm to honeybees can be applicable to humans, thereby leading to the collapse of the Earth's ecosystem."

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