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Research Paper

Comparison of the influence of a pesticide at an environmentally realistic concentration level in Japan on a honeybee colony between neonicotinoids (dinotefuran, clothianidin) and organophosphates (fenitrothion, malathion)

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ABSTRACT

With exception of our previous study on a comparatively high pesticide-concentration level on the assumption that a pesticide is crop-dusted near an apiary there were no reports as regards the differences in the influence on a honeybee (*Apis mellifera*) colony between neonicotinoids and organophosphates. In this paper, the differences between neonicotinoids (dinotefuran and clothianidin) and organophosphates (fenitrothion and malathion) on a realistic pesticide-concentration level in the natural environment surrounding an apiary in Japan was investigated. The long-term field experiment was conducted from August 13th 2013 to April 11th 2014 (241 days). The colonies where the neonicotinoid was administered became extinct after assuming CCD aspect as was the case with our previous three long-term field experiments. It was shown that neonicotinoids can much more rapidly weaken the colony where it was administered than organophosphates and organophosphates can be rapidly degraded in honey stored as a result of their high degradability. These results roughly produced the findings in our previous experiment conducted at higher pesticide-concentrations than this experiment. Such a difference between neonicotinoids and organophosphates can exist in areas ranging from the vicinity of crop-dusted area to the natural environment. Analyzing the pesticide-concentration in residual honey in comb-cells, neonicotinoids whose concentrations in residual honey were lower than those in sugar syrup fed to each colony were detected but organophosphates were hardly detected. It was deduced from the analytical results that organophosphates are decomposed during storing of honey in comb-cells but neonicotinoids are hardly decomposed and that neonicotinoids can continue to be toxic in food (honey and pollen) stored while being diluted by pesticide-free nectar, pollen for a long period of time (during overwintering) and water in fields but organophosphates can rapidly become non-toxic, hence, making it possible for harmless food to be stored in combs. From our research it was inferred that obscure massive colony losses in winter can be probably caused by toxic food with a long-term persistent pesticide such as a neonicotinoid stored in combs during overwintering after the weakening of the colony due to the ingestion of toxic nectar, pollen and water under the natural circumstances contaminated by long-persistent pesticides such as neonicotinoids just before overwintering.

Key words: Dinotefuran, clothianidin, fenitrothion, malathion, neonicotinoid, organophosphate, realistic level, CCD, sugar syrup, field experiment, long-term pesticide, colony, residual concentration, honeybee, overwintering, colony extinction, collapse, acute toxicity, chronic toxicity, *Apis mellifera*.

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INTRODUCTION

After the report of massive losses of honeybee colonies several basic researches on a laboratory level have been performed to investigate the cause of this massive loss and consequently neonicotinoid pesticides (neonicotinoids) have been strongly suspected as a causal agent of these massive losses. Against the opinion from some researchers that a laboratory experiment could not always assign a cause of the massive losses occurring in apiaries, long-term field experiments were conducted for honeybee *Apis millifera* colonies (Lu et al., 2012, 2014; Yamada et al., 2012, 2018a, b). Though the experimental results obtained from Lu et al. (2012, 2014) can give the very rough but significant information on an influence of neonicotinoid pesticides on a honeybee colony, the numbers of adult bees and brood obtained by Lu et al. (2012, 2014) cannot be accurate enough for a scientific data analysis and in consequence it cannot always be affirmed that the experiment was fully reliable.

Yamada et al. (2012, 2018a, b) reported that in order to elucidate the influence of a neonicotinoid pesticide on a honeybee colony focusing on a comparatively high pesticide-concentration level in an apiary not-so-distant from fields where a pesticide is crop-dusted a three long-time field experiments was conducted. The experiments were conducted while endeavoring to obtain the numbers of adult bees and brood as accurately as possible under the conviction that they are most important in understanding the state of a honeybee colony. Yamada et al. (2018b) clarified the difference in the influence on a honeybee colony between two kinds of pesticides of neonicotinoids and organophosphate assuming that they are crop-dusted in fields around the experimental site. Yamada et al. (2018b) inferred that massive losses of honeybee colonies which were hardly incurred before the use of large amount of neonicotinoids could be caused by characteristics such as long-persistency inherent in neonicotinoid. Table 1 shows the results obtained from our previous three works.

The pesticide concentrations were determined to administer to a bee colony in our previous three long-term field experiments on the assumption that honeybees ingest a pesticide on a realistic low-concentration level of a pesticide in the natural environment surrounding an apiary somewhat distant from crop-dusted fields in Japan, based on the following facts: About five ppm of clothianidin was detected in the water near the rice paddy in Hokkaido, Japan (Kakuta et al., 2011), about 0.7 ppm of dinotefuran was detected from paddy fields in Niigata, Japan (Yokoyama et al., 2015) and the maximum residue limits (MRLs) of agricultural chemicals in foods of Japan (JFCRF, 2017) were set at 1 ppm of clothianidin and 2 ppm of dinotefuran in brown rice, broccoli, egg-plant, apple or UNSHU orange and at 25 ppm of clothinidin and 50 ppm of dinotefuran in tea. The experiments were conducted in our apiary where the influence of pesticides due to crop-dusting was slight in Noto district, Hokuriku region, Japan. Neonicotinoids (dinotefuran and clothianidin) and/or organophosphates (fenitrothion and malathion) were used in this work. The pesticide concentrations administered to a bee colony in our previous work (Yamada et al., 2012) were one-tenth of the recommended concentration to exterminate stinkbugs (High), one-fiftieth (Middle) and one-hundredth (Low). The pesticides were administered through toxic sugar syrup and/pollen paste. The pesticides continued to be administered to middle and low colonies till colony extinction or just before overwintering, but were administered for the first time only to high colonies.

Table 1 shows the following findings obtained from the previous works:

(1) In middle and low neonicotinoid colonies, the colonies became extinct after assuming a close similar aspect to Colony Collapse Disorder (CCD) that there were few dead bees around and in their beehives as in the case of control colonies, a queen bee continued to exist with some workers till colony extinction. In high neonicotinoid colonies, heavy mortality of honeybees occurred just after the pesticide administration but high neonicotinoid colonies became extinct while assuming a similar aspect to middle and low neonicotinoid colonies after switching from toxic food containing neonicotinoid to non-toxic food. It was deduced that CCD is not a mysterious phenomenon in a bee colony, but it is only a scene in the process of change in a bee colony which becomes extinct due to a long-persistent pesticide like neonicotinoid.
(2) Organophosphates were extremely hard to make a bee colony become extinct in comparison with neonicotinoids. We infer that the difference on colony extinction between organophosphates and neonicotinoids can come from the persistency of each pesticide. It can be deduced that the toxicity of a pesticide continues to be maintained in toxic food (honey and bee bread) stored in frames of comb (hereafter, combs) over a long period for a neonicotinoid having a long-persistency, but it fades away during the storage in combs for an organophosphate easy to be decomposed into non-toxic compounds.
(3) An examination on an intake route of the neonicotinoid dinotefuran made it clear that the colony which took the pesticide through toxic pollen paste became extinct only at about one-fifth of the pesticide intake through sugar syrup. We infer that the differences of the pesticide intake till colony extinction between sugar syrup and pollen paste seems to be due to the difference of main takers of food (honey or pollen). As honey is taken preferentially by worker bees as energy source but pollen is taken preferentially by brood and a queen bee as protein source and toxic pollen paste containing a neonicotinoid which is long-persistent seems to inflict more serious damage on brood and a queen bee than worker bees.

Encountering criticism that pesticide concentrations are too high in our experiment from some Japanese researchers who advocate a theory that neonicotinoids are not a main cause of colony loss (Nakamura et al., 2014), we planned a long-term field experiment where a pesticide level is one-five hundredths of the recommended concentration to exterminate stinkbugs (very low) for neonicotinoids (dinotefuran and clothianidin) and organophosphates (fenitrothion and malathion) considering the fact that the

Table 1: Previous long-term field experiments conducted by the authors.

Experimental year	Period	Experimental days	Objectives	Pesticide concentration	Experimental results and discussion
2010 (Yamada et al., 2012)	From July, 18th 2010 to Nov. 21st 2010	126	Influence of the neonicotinoid (dinotefuran and clothianidin) on a bee colony and examination of appearance of a CCD phenomenon due to the neonicotinoids	Preparation of each pesticide concentration showing the same insecticidal activity; one-tenth of the concentration to exterminate stinkbugs (High), one-fiftieth (Middle) and one-hundred (Low). High: Dinotefuran (10 ppm), clothianidin (4 ppm) Middle: Dinotefuran (2 ppm), clothianidin (0.8 ppm) Low: Dinotefuran (1 ppm), clothianidin (0.4 ppm). Administration of each pesticide to a colony through both toxic sugar syrup and toxic pollen paste which is prepared by kneading a toxic pollen with toxic sugar syrup. As a result, a pesticide concentration of pollen paste is diluted. To a High colony, a pesticide was administered for the first time.	a) All experimental colonies to which a pesticide was administered became extinct in the end. In Middle and Low colonies, only a few dead bees were observed during experiments. b) In a high colony, massive dead bees were observed near and in a beehive and thereafter only a few bees were observed. c) The experimental colonies became extinct while showing a very similar aspect to CCD. d) Mites were occasionally found just before or after the colony extinction.
2011 (Yamada et al., 2015a)	From July 9th 2011 to April, 2nd 2012	269	Difference in influence of dinotefuran on a bee colony between toxic sugar syrup and toxic pollen paste	Preparation of sugar syrup with dinotefuran concentrations of 10 ppm (High) and 1 ppm (Low): High: Sugar syrup (10 ppm), pollen paste (5.65 ppm) Low: Sugar syrup (1 ppm), pollen paste (0.565 ppm) Administration of dinotefuran to a colony through either toxic sugar syrup or toxic pollen paste.	a) All experimental colonies to which a pesticide was administered became extinct in the end, while roughly duplicating the 2010 experimental results. b) The total intake of dinotefuran per bee to the colony extinction through toxic pollen paste was about one-fifth of that through toxic sugar syrup. We infer that the difference can be due to the fact that pollen is taken mainly by brood and a queen.
2012 (Yamada et al., 2015b)	From June 28th 2012 to July 26th 2013	381	Difference in influence on a bee colony between the neonicotinoid (dinotefuran) and the organophosphate (fenitrothion)	a) Preparation of each pesticide concentration showing the same insecticidal activity; one-fiftieth (Middle) of the concentration to exterminate stinkbugs. b) Where concentrations of dinotefuran and fenitrothion are 2 ppm and 10 ppm in sugar syrup, respectively. Administration of each pesticide to a colony through only toxic sugar syrup.	a) The colony to which dinotefuran (neonicotinoid) was administered became extinct before overwintering. b) On the other hand, the colony to which fenitorothion (organophosphate) was administered succeeded in overwintering in a similar state to the control colony. The success in overwintering of the fenitrothion colony seems to be due to extremely shorter-life persistency and weaker toxicity of fenitrothion (organophosphate) than that of dinotefuran (neonicotinoid).

colony into which fenitrothion was administered succeeded in overwintering as reported by Yamada et al. (2018b).

In this paper, the difference of the chronological changes in the numbers of adult bees, capped brood and dead bees and the analytical results of residual pesticide concentration in honey where a honeybee colony becomes extinct between neonicotinoids and organophosphates at their realistic concentrations in Japan was discussed.

MATERIALS AND METHODS

Materials and preparation of pesticide concentrations

Experiments were conducted from August 13th 2013 to April 11th 2014 (241 days) in the same place as previous field experiments (Latitude 37º1′9″ N, Longitude 165º46′14″ E) under experimental conditions as tabulated in Table 2. STARCKLE MATE10® (10% dinotefuran; Mitsui Chemicals Aglo, Inc., Japan), DANTOTSU® (16% clothianidin; Sumitomo Co. Ltd., Tokyo, Japan), SUMITHION® emulsion (50% fenitrothion; Sumitomo Co. Ltd., Japan) and MALATHON®

Table 2: Outline of experimental conditions in the 2013 experiment (this work).

Item	Detail of experimental conditions
Experimental objective	Difference of the influence on a bee colony between the neonicotinoid and the organophosphate
Kind of pesticide	dinotefuran (STARCKLEMATE 10®, 10% a.i., Mitsui Chemicals Aglo, Inc.); clothianidin (DANTOTSU®, 16 % a.i., Sumitomo Chemical Takeda Agro Co., Ltd.) fenitrothion (SUMITHION emulsion®, 50 % a.i., Sumitomo Co. Ltd.); malathion (MALATHON® emulsion, 50% a.i., Sumitomo Chemical Co., Ltd.)
Experimental period	From August 13th in 2013 (Acclimatization period of a colony: June 27th to August 13th) to April 11th in 2014
Pesticide administration period	From September 5th in 2013 to December 1st for neonicotinoids (dinotefuran, clothianidin) and to a colony extinction during overwintering for organophosphates (fenitrothion, malathion)
Vehicle (food) to administer a pesticide to a colony	Sugar syrup
Concentration of pesticide in sugar syrup	0.2 ppm (dinotefuran) ; 0.08 ppm (clothianidin); 1 ppm (fenitrothion); 1 ppm (malathion)
Frequency of administration of fresh pesticide newly prepared	Only one time (a pesticide was administered to a colony with an auto-feeding system composed of 10 L (14 kg of sugar syrup) container)
Number of colony	Six colonies. Two control colonies (CR-1 and CR-2) which were arranged at the southern end and at the northern end because of the offset of position influence; four experimental colonies which were exposed to dinotefuran (DF), fenitrothion (FT), malathion (MT) and clothinidin (CN) were arranged between two colonies.
Circumstances in an apiary	No crop-dusting within 2 km around, establishment of a new pesticide-free watering place and new plantings of honey crop without the exposure to pesticides in the apiary for experiments
Number of two tiered hive box	Six hives (two controls & four dose tests)
Kind of honeybees	*Apis mellifera*
Initial composition of a hive	Three combs with full bees and some brood and an auto-feeding system with a tank of 10L (sugar syrup=14kg) newly made for this experimental use as shown in Figure 1
Initial number of adult bees and brood at the start of pesticide- administration	Initial number of adult bees in each colony was 5500-7600.and initial number of brood was 4200-5100.
Frequency of observation	At intervals of about one week except winter (When we administered newly-prepared sugar syrup with a pesticide to a colony, we observed all colonies and recorded their conditions by photos on the administration day and the day after). In winter the experiment was conducted in a fine and not-so cold day

Table 2: Conts. Outline of experimental conditions in the 2013 experiment (this work).

Record of colony conditions	Photos of all combs and the inside of a hive with honeybees and of all combs without honeybees taken in every observation
Number of adult bees in a hive	Directly counted with photos of all combs and the inside of a hive one by one after image processing with "Perfect Viewer 7" made by Nanosystem Corporation, Japan
Number of brood in a hive	Directly counted with photos of all combs without honeybees after image processing with "Perfect Viewer 7" made by Nanosystem Corporation, Japan
Number of dead bees	Directly counted in and around a hive one by one with tweezers
Intake of pesticide of honeybees	Accurately weighed by a weighing instrument at the end of experiment
Administration method of pesticide	Administration of toxic sugar by an auto-feeder with 10L-tank (sugar syrup=14 kg) storing them in each hive
Prevention of swarming	Experiment start after the swarming period
Confirmation of a queen bee	Record by photos
Water feeding site	Provide pesticide-free water-feeding site in the apiary
Hornet catcher	Installation of a hornet catcher in each hive after summer
Starting time of each experiment	Early morning except rainy day because of the prevention of a decrease in number due to foraging
Others	Record by photos about troubles such as wax worms, bee-beetles, etc.

emulsion (50% malathion; Sumitomo Co. Ltd., Japan) were used in this work. On comparing the effect of a pesticide on a honeybee colony among them, each pesticide concentration was prepared in order to obtain almost equal insect activity based on each standard concentration of pesticides to exterminate stinkbugs which were representative insect pests in Japan.

Each concentration of pesticides used in this work was determined at the one-five-hundredths of each standard concentration (0.2 ppm for dinotefuran, 0.08 ppm for clothianidin, 1 ppm for fenitrothion and 1 ppm for malathion), which are much lower than a concentration such as about 5 ppm of clothianidin detected near rice fields as reported by Kakuta et al. (2011) and about 0.7 ppm of dinotefuran was reported by Yokoyama et al. (2015). The pesticide concentrations in this work were only one-tenth of those in our previous work (Yamada et al., 2018b). These pesticide concentrations which have the same insecticidal activity affecting stinkbug seem to have almost the same effect on honeybees as reported by Yamada et al. (2012).

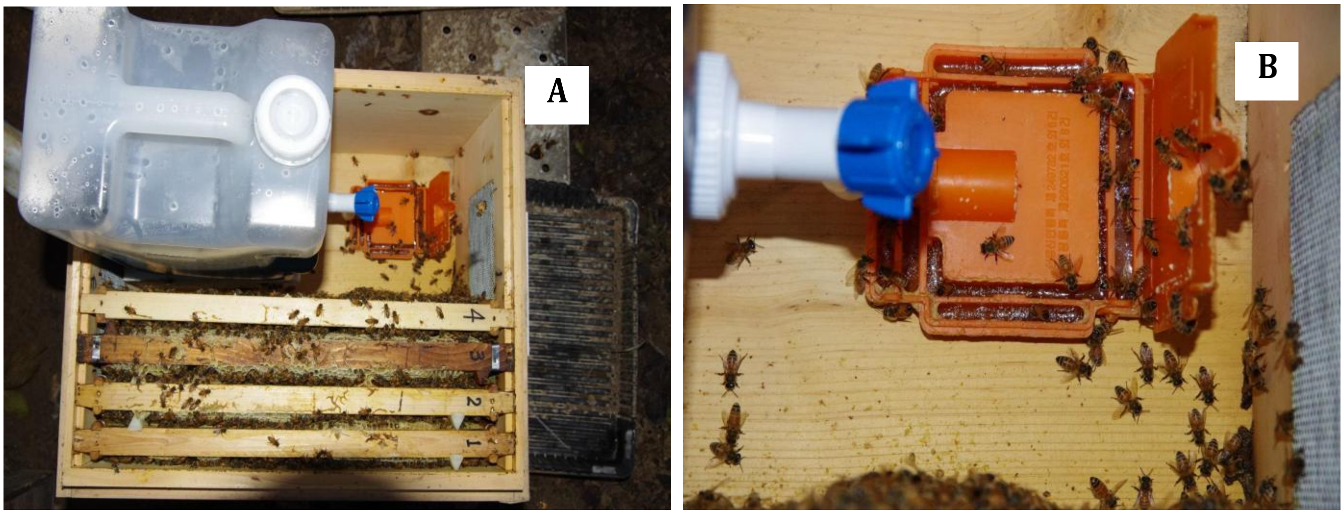

Figure 1: a) Auto-feeding system of sugar syrup solution composed of 10 L (14 kg of sugar syrup) container; b) Intake scene of sugar syrup by honeybees from an auto-feeding system.

Methods used in field experiments

Six beehives, each with 4 numbered combs (frames) and an auto-feeder were sited facing east on a hill. They were aligned in order of the control colony (CR-1) where no pesticide was administered, the dinotefuran colony (DF) where dinotefuran was administered, the fenitrothion colony (FT) where fenitrothion was administered, the malathion colony (MT) where malathion was administered, the clothianidin colony (CN) where clothianidin was administered and another control colony (CR-2) from the south to the north. Two controls were arranged at both ends in order to examine the influence of the difference of location on a bee colony between the northern and southern ends.

Pesticide-free sugar syrup through an auto-feeding system composed of 10 L (14 kg of sugar syrup) container as shown in Figure 1 was fed to eight colonies from June 27th 2013 to the early morning of September 5th as a preliminary experiment in order to acclimatize the colonies to the experimental apiary after the swarming season. We started to conduct the field experiment taking photographs of eight colonies early in the morning on August 13th 2013 and thereafter administered each pesticide into each experimental colonies (DF, FT, MT and CN) early in the morning on September 5th after selecting six colonies from eight colonies. Sugar syrup containing each pesticide and pesticide-free syrup were fed to the experimental colonies and the control colonies through an auto-feeding system composed of 10 L container from the morning of September 5th till the morning of December 1st (just before overwintering) 2013, respectively.

All colonies were observed and every comb taken out of a beehive and put it in another empty beehive after taking photographs of both surfaces of a comb with bees (adult bees) while confirming a queen bee. When we found a queen bee, we took it and then temporarily put it in a queen cage. After taking photographs of every comb with bees and a queen bee, we took an auto-feeder out of the beehive while gently and carefully brushing bees on it down into the beehive. Thereafter, we took photographs of the inside of the beehive (four walls and the bottom) with residual bees. Occasionally, we found the queen bee on the walls or on the bottom. Subsequently, we took photographs of capped brood on both surfaces of every comb without bees (capped brood) after having shaken down bees from a comb and then put it and the auto-feeder back into position. Finally, we gently returned the queen bee into the beehive and took photographs about every week except winter. During overwintering we conducted the experiment without taking photographs of capped brood due to no oviposition with minimum frequency and time in a fine and not so-cold day in order to minimize the damage to a bee colony while preventing a cluster break in opening a beehive. The total number of adult bees on every comb which was numbered and ordered numerically in a beehive and those on the inside of the beehive box (4 walls and bottom) was counted directly and accurately from photographs (sometimes enlarged) of all combs with the help of improved "Perfect Viewer 7" made by Nanosystem Corporation, Japan. The total number of capped brood was counted in a similar manner, after directly shaking the bees off each comb as shown in Figure 2. Though we tried to develop a new automatic counting software with binarizing photograph images (improved "Perfect Viewer 7") which was jointly developed with Nanosystem Corporation, we cannot always succeed in highly accurate counting of them (with an error of not over ± 3%) because it cannot accurately count overlaid bees, bees and capped brood on blurred image and those on low contrast or on low brightness even when

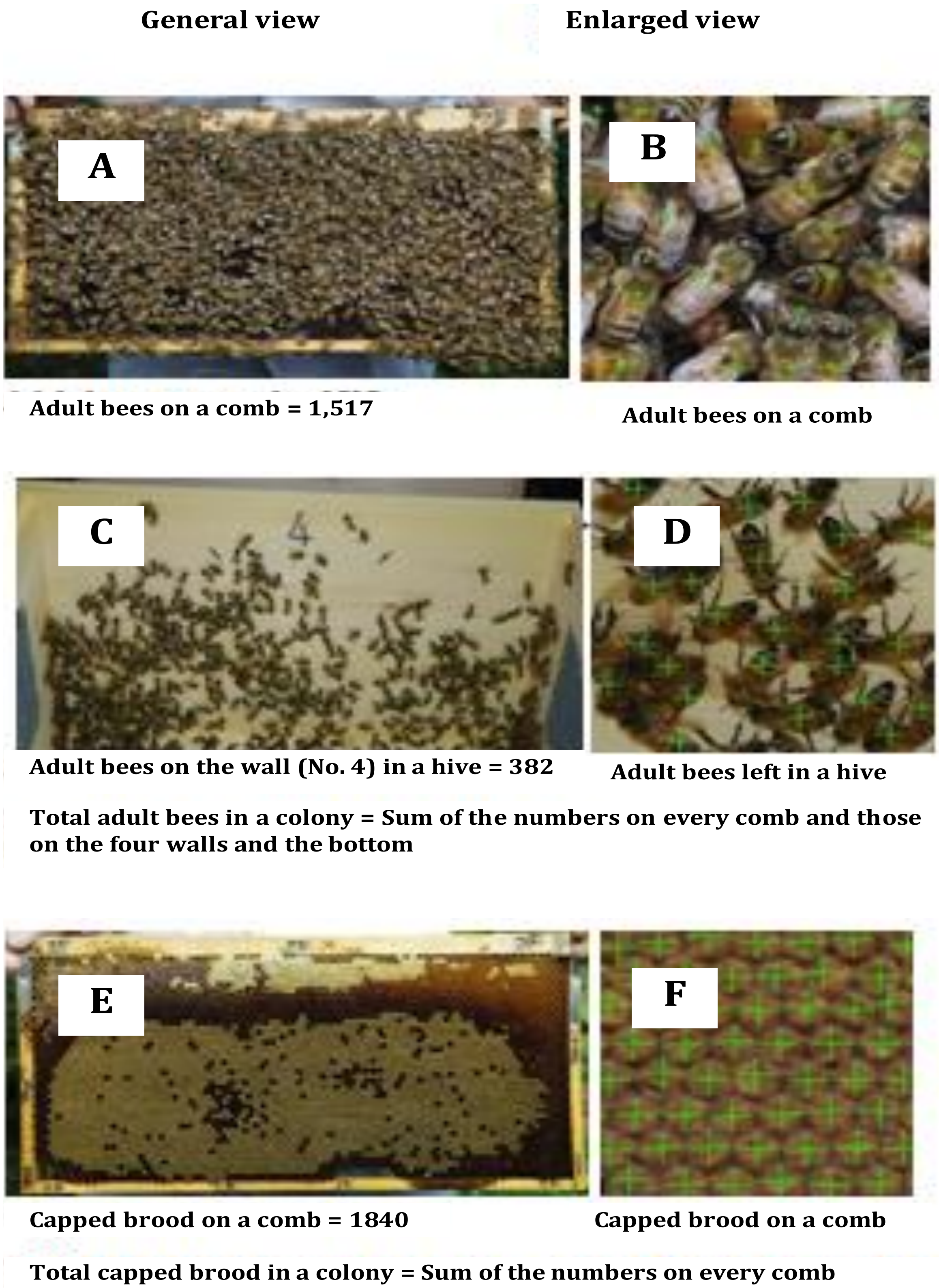

Figure 2: Examples counted in number of adult bees and capped brood.

changing the threshold. The automatic counting software (improved "Perfect Viewer 7") cannot always count the numbers of adult bees and capped brood with high accuracy but it enables reductions in counting time. To

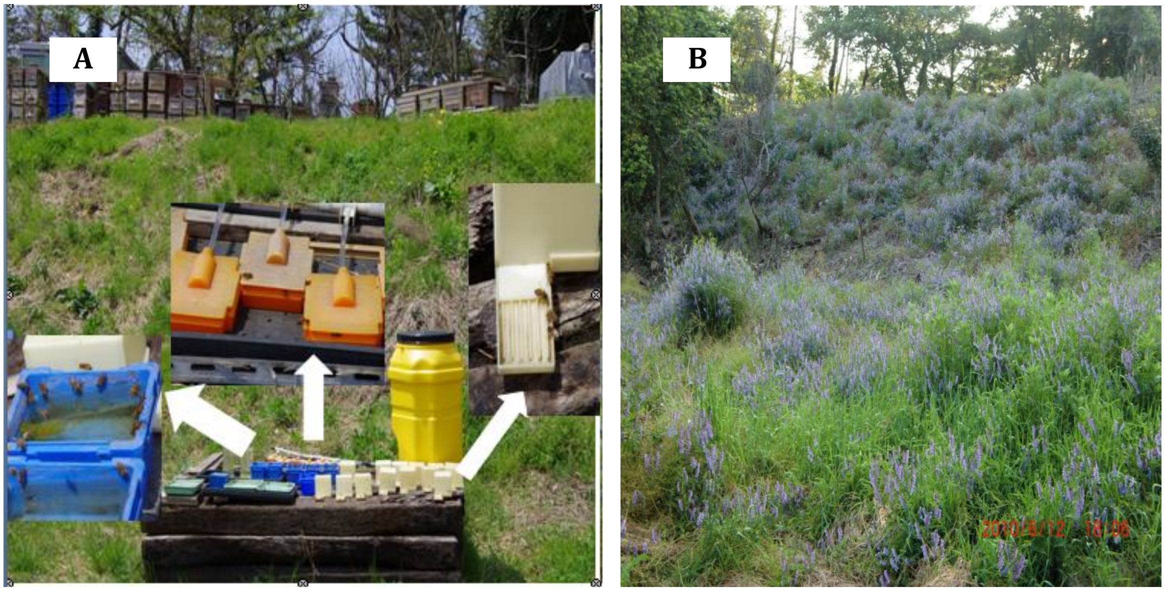

Figure 3: Pesticide-free places in the experimental site (a) Watering place; (b) feeding-place of nectar and pollen. The photo is hairy vetch (*Vicia villosa*) blooming in the experimental site.

obtain the total number of dead bees in and around the beehive and feeder, the beehive was placed on a large tray. The total number of dead bees in the tray, feeder and hive was counted directly, one after the other with a pair of tweezers.

The queen bee in the beehive was photographically recorded on each measurement date, as were specific situations such as the presence of chalk brood or wax moth larvae and the evidence of Asian giant hornet attacks. During the experimental period, each hive state was automatically recorded at intervals of 1 h with a digital camera.

The experiment was conducted the early in the morning on fine or cloudy days from August 13th 2013 to April 11th 2014, before the foraging bees left the beehive. In order to decrease in unclearness and diversity of uncontrollable factors contained in field experiments, we selected an experimental site where there are no aerial-sprayed paddy fields and orchards in the vicinity. A honeybee-watering place was newly located in the experimental apiary in order to supply honeybees with pesticide-free water. Pesticide-free pollen and nectar were supplied following organically-grown plants around the experimental site so as to prevent honeybees from taking nectar and pollen contaminated by pesticides with the intention to minimize the effects of environmental factors such as leaf mustard (*Brassica juncea*), hairy vetch (*Vicia villosa*), Japanese chestnut-trees (*Castanea crenata*) and fruit-trees such as citrus trees and persimmon trees (*Diospyros kaki*) etc (Figure 3).

The consumption of sugar syrup by honeybees was accurately measured by a weighing instrument having an accuracy of 0.1 g in every observation. The net intake of a pesticide was obtained from the amount of sugar syrup consumed by honeybees. The cumulative total intake of each active ingredient (dinotefuran, clothianidin, fenitrothion and malathion) was obtained from the amount of sugar syrup consumed by a honeybee colony during the pesticide-administration period from September 5th to December 1st. The interval intake of a pesticide by a honeybee colony between two observation dates (a certain observation date and the previous date) was obtained from the consumption of sugar syrup with a pesticide. Considering that sugar syrup with a pesticide before overwintering was stored in cells in combs as honey, honeybees probably continued to ingest the pesticide through honey stored in cells during the overwintering period when no sugar syrup was administered. The intake of a pesticide per bee was estimated from dividing the cumulative total intake of the pesticide in a honeybee colony by the sum of the total number of newborn honeybees, the initial number of honeybees at the start of experiment and the number of the capped brood finally existing in a honeybee colony at the colony extinction.

Strictly speaking, this work cannot be always conducted under the very same conditions as natural environment near an actual apiary, because sugar syrup is not same as nectar in fields and the feeding area in this work is not the same as that in an actual apiary. That is, honeybees in an experimental colony of this work take not only toxic sugar syrup in a beehive but also nectar which is controlled mainly so as to be non-toxic by pesticide-free flowers in an apiary, while those in a honeybee colony of an actual apiary take nectar which is toxic and/or non-toxic in fields. In addition, not only foraging bees but also house bees may take sugar syrup in this work, while only foraging bees take nectar in fields in an actual apiary. Despite these differences between an actual apiary and this work, we believe that this work can possibly replicate most of the phenomena occurring in an actual apiary though we have to give maximum attention to them.

RESULTS

Long-term observations

The experiment was conducted under the nearly natural environment where honeybees can freely take foods in fields if they do not like to take toxic sugar syrup in a beehive. Table 3 shows the observational summary of this work (2013 experiment) from August 13th 2013 to April 11th 2014, which lists the administration of a pesticide, the existence of a queen bee, the number of queen cells and the number of frames in a beehive and besides, remarks in observation.

Table 3 shows that the queen bee of every colony existed just before extinction and no queen bee cell existed in a bee colony except dinotefuran colony (DF) where a few queen cells were produced on combs and thereafter a new queen bee was born after the old one had been lost. We observed that both of the neonicotinoid colonies (DF and CN) went into extinction on January 5th 2014; one (CR-1) of the control colonies (CR-1 and CR-2) and one (MT) of organophosphate colonies (FT and MT) became extinct on February 7th 2014, while another organophosphate colony (FT) became extinct on February 28th 2014. Only one control colony survived at the end of the experiment. We infer that a cluster break in opening a beehive to take photographs in winter may possibly have triggered the extinction of the control colony (CR-1) and organophosphate colonies (FT and MT) judging from the fact that the fenitrothion colony had succeeded in overwintering as was the case with the control colony in our previous work (Yamada et al., 2018b).

Measurement of number of adult bees and capped brood

Table 4 shows the numbers of adult bees and capped brood in this work. Pesticide-free sugar syrup was fed into every colony from August 13th to the early morning of September 5th 2013 and immediately sugar syrup containing a pesticide (dinotefuran, clothianidin, fenitrothion and malathion) began to be fed into the experimental colonies (DF, CN, FT and MT), respectively. We started to conduct the experiment where we took photographs, counted dead bees in and around a beehive; counted queen cells and wax worms in a beehive confirmed the queen bee and observed the state of a honeybee colony such as colony activity and bee-diseases from August 13th 2013. The elapsed days in Table 4 shows the number of days elapsed from the start of the experiment (August 13th), which are not the number of days from the start of pesticide administration (September 5th).

Figure 4 shows the seasonal change in the number of adult bees from August 13th 2013 to February 28th 2014. The numbers of adult bees of two neonicotinoid colonies (DF and CN) analogously changed with day while being kept smaller than those of the other colonies (CR-1, CR-2, FT and MT). The numbers of adult bees of the neonicotinoid colonies just before overwintering on December 1st 2013 were considerably smaller than those of the other colonies. The changes in the numbers of adult bees of organophosphate colonies (FT and MT) are similar to those of the control colonies as earlier described in our previous work (Yamada et al., 2018b). These facts suggest that neonicotinoids seems to exert a baneful influence on a bee colony but organophosphates seem to exert a little adverse influence on a bee colony. Neonicotinoids especially, have a severe effect on a bee colony both acutely and chronically but organophosphates do only acutely. Both of the neonicotinoid colonies (DF and CN) had already become extinct when the colonies were observed in January 5th 2014 (first observation during overwintering). One (CR-1) of the control colonies and one (MT) of the organophosphate colonies became extinct on February 7th and another one (FT) of the organophosphate colonies became extinct on February 28th. There is a suspicion that the opening of a beehive to observe a bee colony during overwintering had an adverse effect on a bee colony such as a cluster break. Only one colony (CR-2) succeeded in overwintering.

Figure 5 shows the seasonal change in the number of capped brood from August 13th 2013 to February 28th 2014. The number of capped brood in every colony is approximately zero during overwintering. The numbers of capped brood of both neonicotinoid colonies (DF and CN) were smaller than those of the other colonies (CR-1, CR-2, FT and MT) and approached zero earlier than those of the other colonies. These results suggest that neonicotinoids inhibit the laying of eggs and accelerate the stop of egg-laying for overwintering. On the other hand, the numbers of capped brood of the organophosphate colonies (FT and MT) changed with day as was the case with the control colonies (CR-1 and CR-2). There was no capped brood in winter in all the colonies.

Measurement of the number of dead bees

We measured the interval number of dead bees in an interval between two adjacent observation dates existing inside (on the bottom and in a feeder) and outside (mainly in the front) of the beehive. Table 5 shows the interval number of dead bees at every observation date. These results were illustrated in Figure 6 after the conversion of the interval number of dead bees between two adjacent

Table 3: Observational summary in this work from August 13th to April 11th 2014.

Date	Starting time	Elapsed days		Control-1 colony (CR-1)				Dinotefuran colony (DF)				Fenitrothion colony (FT)				Malathion colony (MT)				Clothianidin colony (CN)				Control-2 colony (CR-2)				Remarks
		from the start of experiment	from pesticide administration	Pesticide	Queen	NO of queen cells	NO of frames	Pesticide	NO of Queen queen cells		NO of frames	Pesticide	Queen	NO of queen cells	NO of frames	Pesticide	Queen	NO of queen cells	NO of frames	Pesticide	Queen	NO of queen cells	NO of frames	Pesticide	Queen	NO of queen cells	NO of frames	
Concentration of pesticide administered to a colony				Pesticide-free				0.2 ppm (dinotefuran)				1 ppm (fenitrothion)				1 ppm (malathion)				0.08 ppm (clothianidin)				Pesticide-free				
We selected six colonies for the experiment which resembles each other in ecology activity among twelve colonies after the preparation rearing period between June 27th in 2013 and August 13th																												
13-Aug-13	5:00	0		free	present	0	4	free	present	0	4	free	present	0	4	free	present	0	4	free	present	0	4	free	present	0	4	1) Start of the experiment (taking photos)
24-Aug-13	5:40	11		free	present	0	4	free	present	0	4	free	present	0	4	free	present	0	4	free	present	0	4	free	present	0	4	
1-Sep-13	5:40	19		free	present	0	4	free	present	0	4	free	present	0	4	free	present	0	4	free	present	0	4	free	present	0	4	
5-Sep-13	5:30	23	0	free	present	0	4	dinotefuran (0.2 ppm)	present	0	4	fenitrothion (1 ppm)	present	0	4	malathion (1 ppm)	present	0	4	clothianidin (0.08 ppm)	present	0	4	free	present	0	4	1) Start of the pesticide administration 2) An equipment to capter a hornet is installed at the front of each beehive
5-Sep-13	16:30	23		free			4	dinotefuran (0.2 ppm)			4	fenitrothion (1 ppm)			4	malathion (1 ppm)			4	clothianidin (0.08 ppm)			4	free			4	1) Only the number of dead bees of each beehive was counted without taking photos.
6-Sep-13	9:30	24	1	free			4	dinotefuran (0.2 ppm)			4	fenitrothion (1 ppm)			4	malathion (1 ppm)			4	clothianidin (0.08 ppm)			4	free			4	2) Only the number of dead bees of each beehive was counted without taking photos. 3) Each auto-feeding system is checked and no problems were found.
15-Sep-13	5:40	33	10	free	present	0	4	dinotefuran (0.2 ppm)	present	0	4	fenitrothion (1 ppm)	present	0	4	malathion (1 ppm)	present	0	4	clothianidin (0.08 ppm)	present	0	4	free	present	0	4	1) The experiment was suspended because of rain after taking the photos of CR-1 colony and it resumed at 11:40. 2) A wax moth *lesser Achroia griella* was in each colony of CR-1 & FT.
21-Sep-13	5:30	39	16	free	present	0	5	dinotefuran (0.2 ppm)	present	0	4	fenitrothion (1 ppm)	present	0	4	malathion (1 ppm)	present	0	4	clothianidin (0.08 ppm)	present	0	4	free	present	0	5	1) There were evidences that every colony except CR-2 was attacked by giant hornets. 2) A few burr-combs were found in CR-1. 3) A few wax moths *lesser Achroia griella* were in every colony except CR-2.
27-Sep-13	5:45	45	22	free	present	0	5	dinotefuran (0.2 ppm)	present	0	4	fenitrothion (1 ppm)	present	0	4	malathion (1 ppm)	present	0	4	clothianidin (0.08 ppm)	present	0	4	free	present	0	5	1) There were evidences that every colony was attacked by giant hornets. 2). A few wax moths l*esser Achroia griella* were in every colony except CR-1 &CR-2.

Table 3: Conts. Observational summary in this work from August 13th to April 11th 2014.

4-Oct-13	5:45	52	29	free	present	0	5	dinotefuran (0.2 ppm)	present	0	4	fenitrothion (1 ppm)	present	0	4	malathion (1 ppm)	present	0	4	clothianidin (0.08 ppm)	present	0	4	free	present	0	5	1) There were evidences that every colony (especially, MT & CN) except CR-2 was greatly attacked by giant hornets.
13-Oct-13	6:00	61	38	free	present	0	5	dinotefuran (0.2 ppm)	absent	3	4	fenitrothion (1 ppm)	present	0	4	malathion (1 ppm)	present	0	4	clothianidin (0.08 ppm)	present	0	4	free	present	0	5	There were evidences that every colony (especially, CR-1 & MT) except CN & CRL-2 was attacked by giant hornets. 2) A few wax moths *lesser Achroia griella* were in every colony. 3) DF was queenless and had three small queen cells.
18-Oct-13	6:05	66	43	free	present	0	4	dinotefuran (0.2 ppm)	absent	2	4	fenitrothion (1 ppm)	present	0	4	malathion (1 ppm)	present	0	4	clothianidin (0.08 ppm)	present	0	4	free	present	0	4	1) There were evidences that every colony except DF & CN was slightly attacked by giant hornets. 2) DINOTEFURAN was queenless and had two queen cells (one of three disappeared). 3) No drone was found in every colony.
27-Oct-13	6:00	75	52	free	present	0	4	dinotefuran (0.2 ppm)	present (new queen)	0	4	fenitrothion (1 ppm)	present	0	4	malathion (1 ppm)	present	0	4	clothianidin (0.08 ppm)	present	0	4	free	present	0	4	1) The experiment was suspended because of rain and it resumed at 7:00. 2) There were few evidences that every colony was attacked by giant hornets. 3) A new queen was put in DF because the loss of the queen may not be due to an affect of dinotefuran but it may be due to a common accident among honeybee colonies.
15-Nov-13	12:30	94	71	free	present	0	4	dinotefuran (0.2 ppm)	present	0	4	fenitrothion (1 ppm)	present	0	4	malathion (1 ppm)	present	0	4	clothianidin (0.08 ppm)	present	0	4	free	present	0	4	1) The experiment was started at 12:30 because of rain. 2) A new queen in DF was right.
1-Dec-13	8:30	110	87	free	present	0	4	dinotefuran (0.2 ppm)	present	0	4	fenitrothion (1 ppm)	present	0	4	malathion (1 ppm)	present	0	4	clothianidin (0.08 ppm)	present	0	4	free	present	0	4	1) The experiment was started at 8:30 because of rain and cold (temperature is 8℃). 2) As capped brood were not found in every colony, the photos without honeybees were not taken. 3) Preparation to overwinter every colony was made by coverinhg the outside of each hive with heat-insulating material. 4) Pesticide-free sugar syrup was fed into every colony.

Table 3: Conts. Observational summary in this work from August 13th to April 11th 2014.

5-Jan-14	9:30	145	122		present	0	4		absent (colony extinction)	0	4		present	0	4		present	0	4		absent (colony extinction)	0	4		present	0	4	1) The experiment was started at 9:30 because of cold (temperature is 4 ℃). 2) As capped brood were not found in every colony, the photos without honeybees were not taken. 3) DF & CN (neonicotinoids) had already become extinct but FT & MT (organophosphates) survived though a queen in MT was dead. It is feared that the clusters of the surviving colonies may be broken by openning the beehives and take out the combs in the cold to take photos.
7-Feb-14	11:30	178	155		absent (colony extinction)	0	0						present	0	4		absent (colony extinction)	0	4						present	0	4	1) The experiment was started at 11:30 because of cold (temperature is 2 ℃). 1) As capped brood were not found in every colony, the photos without honeybees were not taken. 2) CR-1 & MT had become extinct. The extinctions of CR-1 & MT maybe caused by the cluster break and debility of a colony due to the great temperature change and/or the exposure of combs to the cold outside air to take photos on January 5th.
28-Feb-14	8:30	199	176										absent (colony extinction)	0	4										present	0	4	1) CR-2 survived, but FT had become extinct. The extinction of FT also may be caused by the cluster break and debility due to the great temperature change and/or the exposure of combs to the cold outside air to take photos on January 5th and February 7th. 2) As capped brood were not found in CR-2, the photos without honeybees were not taken.
11-Apr-14	10:30	241	218																						present	0	4	1) It was confirmed that CR-2 succeeded in overwintering by visual inspections. The photos of adult bees & brood were not taken.

Note 1: It is feared that taking photos while opening a beehive in winter makes the breakup of a bee-cluster accelerate. Note 2: Sugar syrup containing each pesticide was fed to the experimental colonies from the morning of September, 5th till the morning of December, 1st 2013. Note 3: Pesticide-free sugar syrup was fed to the control colonies from the morning of September, 5th till the morning of December, 1st 2013. Note 4: The extinctions of Control-1, Fenitrothion and Malathion colonies may be due to cluster-breakup in the observational experiment on January, 5th 2013.

Table 4: Number of adult bees and brood in this work.

Date	Elapsed days	CR-1		CR-2		DF 0.2 ppm		CN 0.08 ppm		FT 1 ppm		MT 1 ppm	
		Adult	Brood	Adult	Brood	Adult	Brood	Adult	Brood	Adult	Brood	Adult	Brood
13-Aug-13	0	7579	4167	5953	4683	5447	4605	5628	5022	7360	5094	5500	4616
24-Aug-13	11	7000	3363	6041	2146	7556	323	6915	2208	9290	3758	6803	2414
1-Sep-13	19	7324	1825	5565	558	6529	1219	7790	1025	10402	2671	8177	1054
5-Sep-13	23	7487	2261	5549	578	6017	2214	7293	1506	10296	3232	7676	1396
15-Sep-13	33	6415	5462	4027	2665	5072	1743	5574	1825	5795	6040	5716	4992
21-Sep-13	39	6697	4683	3829	3198	5154	90	4001	843	7431	3015	5698	3943
27-Sep-13	45	5711	2538	3889	2882	3317	0	3885	208	6920	1655	5617	2456
4-Oct-13	52	5048	649	3824	2142	3145	0	1882	160	5124	1456	4445	984
13-Oct-13	61	4210	151	4322	113	2029	0	1762	7	4478	11	3459	0
27-Oct-13	75	3152	314	3371	0	1325	0	1449	0	3147	0	2328	0
15-Nov-13	94	2714	50	3181	0	1146	0	1143	0	2554	0	2050	0
1-Dec-13	110	2189	3	2904	0	795	0	867	0	2213	0	1745	0
5-Jan-14	145	1100	0	2218	0	0	0	0	0	1714	0	916	0
7-Feb-14	178	0	314	1799	0					878	0	0	0
28-Feb-14	199	0		1594	0					0	0		

Note 1: Pesticide was administered from September, 5th 2013. Note 2: All of neonicotinoids (DF and CN) became extinct on January, 5th 2014. Note 3: CR-1and MT became extinct on February, 7th 2014. Note 4: FT became extinct on February, 28th 2014. Only CR-1 succeeded in overwintering. Note 5: Capped brood contained both brood in case where more than 80% (r/R=0.45) of a cell area is capped and brood just before eclosion. Note 6: R is a radius of a cell, while r is a radius of a capless part of a cell.

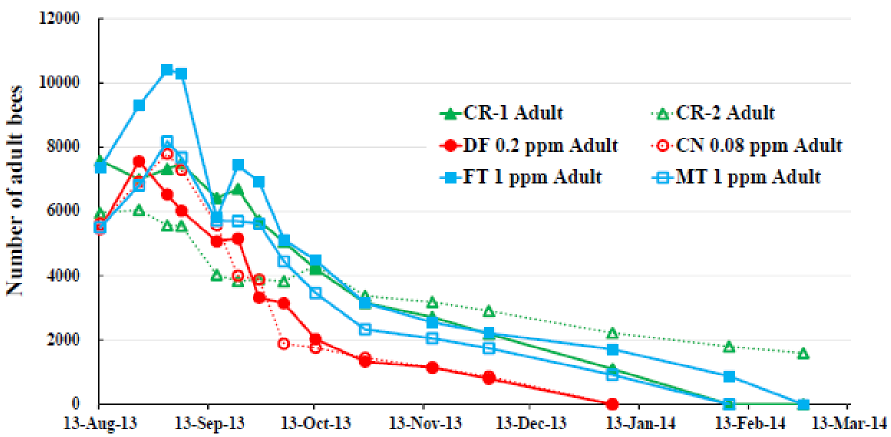

Figure 4: Seasonal change in the number of adult bees from August 13th in 2013 to February 28th in 2014. Each pesticide was administered from September 5th to December 1st in 2013.

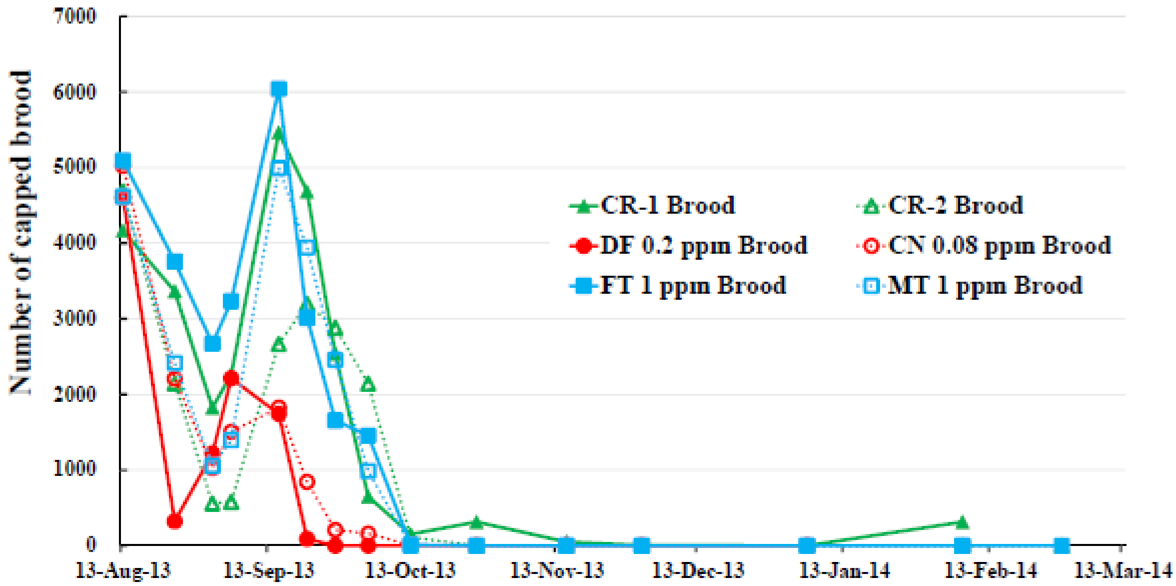

Figure 5: Number of capped brood.

Table 5: Mean number of dead bees per day in an interval between two adjacent observations in this work.

Date	CR-1	CR-2	DF	CN	FT	MT
3-Aug-13	0	0	0	0	0	0
13-Aug-13	0.2	0.3	1.7	0	9	0.8
24-Aug-13	1.18	3.73	1.18	1.91	1.64	5.36
1-Sep-13	0.13	0	0.13	0.88	0.25	3.25
5-Sep-13	3.5	2.25	2.5	4.5	2.25	3
5-Sep-13	4	110	0	24	6	8
6-Sep-13	0	5	2	7	1	1
15-Sep-13	0	0.222	1.89	0.11	15.78	0.33
21-Sep-13	31.83	0.33	5.17	102.83	9.17	12.67
27-Sep-13	150.83	0.833	161.83	2.83	21.17	33.67
4-Oct-13	41.86	3	9.14	13.43	59.43	15.14
13-Oct-13	2.44	0.44	1.67	0.33	9.33	24
18-Oct-13	34	30.6	1.8	2.8	25.8	21
27-Oct-13	3.67	0.78	2.89	2.33	10.11	13
15-Nov-13	2.79	1.26	1.37	3.58	0.53	1.58
1-Dec-13	4	2.13	4.5	9.56	1.06	3.56
5-Jan-14	8.14	2.43			7.57	6.71
7-Feb-14	90.91	3.48			21.36	0.24
28-Feb-14		10.48			142.86	

Note: The number of dead bees was obtained by summing up the numbers around and inside a beehive.

observations into the number of dead bees per day (daily number of dead bees). Table 5 and Figure 6 show that few dead bees could be found in and around a beehive except that several dead bees were found when a honeybee colony was attacked by Asian giant hornets or was just about to become extinct.

Measurement of the number of mites and wax worms in a bee colony

We counted the number of mites and wax worms in a bee colony while visually examining a bee-colony (combs and the inside of a beehive) with great care during an

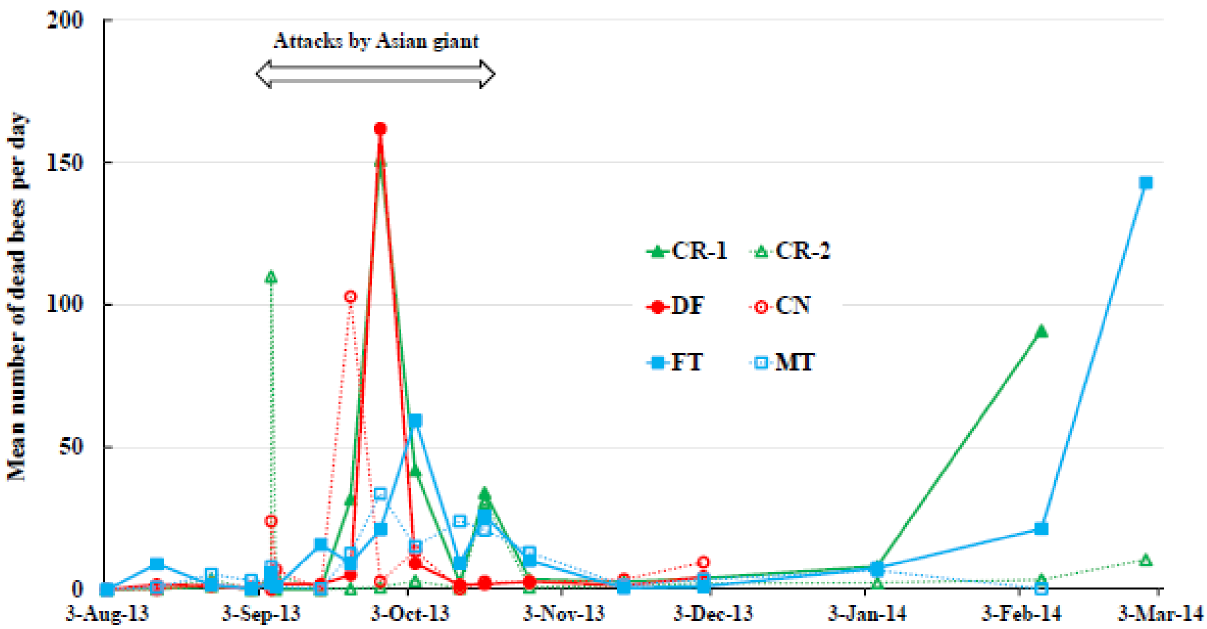

Figure 6: Daily number of dead bees near and inside the beehive.

experiment and while scrutinizing each enlarged photograph carefully after the experiment where the number of wax worms includes that of wax moths. Table 6 shows the numbers of mites and wax worms (moths). We can find that mites and wax worms (wax moths) cannot exist before colony extinction.

Intake of pesticide by a honeybee colony

Table 7 shows the intake of a pesticide per colony and that per bee, intake of sugar syrup per colony and that per bee and converted intake of a pesticide into its equivalent clothianidin per colony and that per bee till colony extinction in 2013 experiment. The intake per colony of sugar syrup in the control colonies (CR-1 and CR-2) are more than the experimental colonies (DF, CN and FT) with exception of MT. Pesticides such as neonicotinoids and organophosphates may possibly diminish the intake of sugar syrup. Roughly speaking, the intake per colony of a pesticide in the neonicotinoid colonies (DF and CN) differs little from that in the organophosphate colonies (FT and MT), though the intake in DF is a little much less and that in MT is a little much more.

Secondly, we examined the intake per bee of a pesticide and sugar syrup. In order to obtain the intake per bee, we need to obtain the total number of adult bees which are newly emerging from capped brood (pupae). The total number of adult bees in the experimental colonies (DF, CN, FT and MT) was calculated in order to discuss the intake of a pesticide per bee till colony extinction according to the procedures proposed by Yamada et al. (2018a, b). The total numbers of adult bees calculated in DF, CN, FT and MT are 8612, 10130, 19585 and 15723 heads, respectively. These colonies became extinct in the reverse order of total number. Judging from the fact that hardly any capped brood exists and few adult bees newly emerge during overwintering, the total number of adult bees in a honeybee colony may depend largely on the success in overwintering.

Currently, we can obtain the intake of each pesticide per bee when dividing the total intake of a pesticide by the total number of adult bees. Table 7 shows each pesticide intake per bee is 59.69 ng/bee in DF, 39.64 in CN, 197.09 in FT and 429.29 in MT. Every intake of a pesticide seems to be enough amount for a bee colony to collapse judging from the median lethal dose (LD_{50}) which is 7.6 to 32 ng/bee [dinotefuran] for oral acute and 24 to 61 ng/bee [dinotefuran] for contact (U.S.EPA, 2004), 47 ng/bee [dinotefuran] for acute contact (Durkin, 2009), 75 ng/bee [dinotefuran] for contact (Iwasa et al., 2004), > 0.023 ng/bee for contact acute/48 h (PPDB, 2016a), 44 ng/bee [clothianidin] for contact (U.S.EPA, 2004), 22 ng/bee [clothianidin] for contact (Iwasa et al., 2004), 22 to 44 ng/bee [clothianidin] for contact and 2.8 to 3.7 ng/bee [clothianidin] for oral acute (Hodgson, 2012), 3.53 to 3.28 ng/bee [clothianidin] for oral acute in 24 to 72 h (Laurino et al., 2013), 4 ng/bee [clothianidin] for oral acute (Sanchez-Bayo, 2014), 26.9 to 9.5 ng/bee [clothianidin] for oral acute in 48 to 240 ho (Alkassab and Kirchner, 2016), 44 ng/bee [clothianidin] for contact acute in 48 h and 4 ng/bee [clothianidin] for oral acute in 48 h (PPDB, 2016b); 18 ng/bee [fenitrothion] (NUFARMNZ, 2012), 30 to 40 ng/bee [fenitrothion] for contact (Wang et al., 2012), 160 ng/bee [fenitrothion] for contact and 200 ng/bee [fenitrothion] for oral acute (PPDB, 2017a), 176 ng/bee

Table 6: Numbers of mites and wax worms obtained from all photos enlarged and direct observation in this work.

Date	Elapsed days	CR-1		DF 0.2 ppm		FT 1 ppm		MT 1 ppm		DF 0.2 ppm		CR-2	
		Mite	Wax worm	Mite	Wax worm	Mite	Wax worm	Mite	Wax worm	Mite	Wax worm	Mite	Wax worm
13-Aug-13	0	0	0	0	0	0	0	0	0	0	0	0	0
24-Aug-13	11	0	0	0	0	0	0	0	0	0	0	0	0
1-Sep-13	19	0	0	0	0	0	0	0	0	0	0	0	0
5-Sep-13	23	0	0	0	0	0	0	0	0	0	0	0	0
15-Sep-13	33	0	0	0	0	0	0	0	0	0	0	0	0
21-Sep-13	39	0	0	0	0	0	0	0	0	0	0	0	0
27-Sep-13	45	0	0	0	0	0	0	0	0	0	0	0	0
4-Oct-13	52	0	0	0	0	0	0	0	0	0	0	0	0
13-Oct-13	61	0	0	0	0	0	0	0	0	0	0	0	0
27-Oct-13	75	0	0	0	0	0	0	0	0	0	0	0	0
15-Nov-13	94	0	0	0	0	0	0	0	0	0	0	0	0
1-Dec-13	110	0	0	0	0	0	0	0	0	0	0	0	0
5-Jan-14	145	0	0	0	0	0	0	0	0	0	0	0	0
7-Feb-14	178	0	0			0	0	0	0			0	0
28-Feb-14	199	0	0			0	2					0	0

Note: The number of wax worm includes that of wax moth in a bee colony.

[fenitrothion] for contact (Sanford, 2003), 200 ng/bee [fenitrothion] for oral acute and 160 ng/bee [fenitrothion] for acute contact (WHO, 2010); 270 ng/bee [malathion] for acute contact and 380 ng/bee [malathion] for acute oral (Stevenson, 1978; WHO, 2003), 335.2 ng/bee [malathion] for acute oral (Hardstone and Scott, 2010), 160 ng/bee [malathion] for acute contact in 48 h and 400 ng/bee [malathion] for acute oral in 48 h (PPDB, 2017b). As the LD_{50} (50% lethal dose) which is one way to measure the short-term poisoning potential of a pesticide, we should pay attention to the fact that it can give the useful information on the death of honeybees due to the acute toxicity in a short-term dose but cannot always estimate a honeybee colony extinction due to the chronic toxicity in a long-term dose.

To compare the insecticidal ability of each pesticide intake among experimental colonies, we expressed the each pesticide intake by converting it into the intake of clothianidin having almost the same insecticidal activity to exterminate stink-bugs as it, where the insecticidal activities of dinotifuran, fenitrothion and malathion are 0.4 times, 0.08 times and 0.08 times as much as that of clothianidin, respectively. The intake of each pesticide converted into the intake of clothianidin (the converted intake of each pesticide) per bee till colony extinction is 23.88 ng/bee for dinotefuran, 39.64 ng/bee for clothianidin, 15.77 ng/bee for fenitrothion and 34.34 ng/bee for malathion, respectively, as shown in Table 7. Though the converted intake of fenitrothion per bee till colony extinction is somewhat less than those of the other pesticides, it seems to be enough to extinct the colony (FT) in a long-term dose.

Concentration of residual pesticide in honey

Table 8 shows the concentration of residual pesticide in honey stored in combs of an extinct colony. It has been found from the analysis of the residual honey in the extinct colony that the concentrations of dinotefuran, clothianidin, fenitrothion and malathion are 0.045 ppm, 0.033 ppm, 0.01 ppm and not detected (0.00 ppm), respectively. These concentrations were measured with liquid chromatography tandem-mass spectrometry (LC-MS/MS) by Saika Technologies, Inc., Japan. From these results, we observed that

Table 7: Intake of a pesticide per colony and per bee, intake of sugar syrup per colony and per bee and converted intake of a pesticide into its equivalent clothianidin per colony and per bee till colony extinction in this work.

Kind of colony	**CR-1**	**CR-2**	**DF (0.2 ppm)**	**CN (0.08 ppm)**	**FT (1 ppm)**	**MT (1 ppm)**
Kind of pesticide	**Pesticide free**	**Pesticide free**	**Dinotefuran**	**Clothianidin**	**Fenitrothion**	**Malathion**
Intake of a pesticide per colony [mg]	-	-	0.514	0.4016	3.86	6.75
Intake of sugar syrup per colony [g]	11840	8590	2570	5020	3860	8750
Total number of adult bees per colony till colony extinction	-	-	8611.83	10130.33	19585.25	15723.5
Intake of a pesticide per bee till extinction [ng/bee]	-	-	59.69	39.64	197.09	429.29
Intake of sugar syrup per bee till colony extinction [g/bee]	-	-	0.2984	0.4955	0.1971	0.5565
Converted intake of a pesticide into its equivalent clothianidin per colony till colony extinction [mg]	-	-	0.2056	0.4016	0.3088	0.54
Converted intake of a pesticide into its equivalent clothianidin per bee till colony extinction [ng/bee]	-	-	23.88	39.64	15.77	34.34

The concentration of dinotefuran, that of clothianidin, that of fenitrothion and that of malathion recommended by a manufacturer of agrochemicals to exterminate stinkbugs are 100 ppm, 40 ppm, 500 ppm and 500ppm, respectively. Therefore, the insecticidal activities of dinotefuran, fenitrothion and malathion are estimated to be 0.4 times, 0.08 times and 0.08 times as much as that of clothianidin, respectively. Converted intake of each pesticide into its equivalent clothianidin per colony or per bee till colony extinction can be calculated from the following relation: Intake of a pesticide per colony or per bee × 0.4 for dinotefuran and 0.08 for fenitrothion and malathion, respectively. For example, the converted intake of dinotefuran into its equivalent clothianidin per colony or per bee is 0.514 mg × 0.4 = 0.2056 mg in clothianidin (per colony) or 59.69 ng/bee × 0.4 = 23.88 ng/bee in clothianidin (per bee).

Table 8: Concentration of residual pesticide in honey stored in an extinct colony in this work.

Kind of pesticide	**Neonicotinoid**		**Organophosphate**	
	Dinotefuran	**Clothianidin**	**Fenitrothion**	**Malathion**
Conc. of pesticide in sugar syrup fed to a colony (A)	0.2	0.08	1	1
Conc. of pesticide when sugar syrup (50 wt% water) fed to a colony is converted into honey (20 wt% water) (A') [ppm]	0.32	0.128	1.6	1.6
Conc. of residual pesticide in honey stored in combs of a extinct colony (B) [ppm]	0.045	0.033	0.01	N.D. (0.00)
Apparent ratio of residual pesticide in a hive to administered one to a colony (B/A) [-]	0.225	0.413	0.01	0
Ratio of pesticide in honey left in an extinction colony to conc. of pesticide in sugar syrup converted into honey fed to a colony (B/A') [-]	0.141	0.234	0.006	0
Limit of quantification (LOQ) [ppm]	0.01	0.005	0.01	0.01

When sugar syrup (50 wt% water) fed to a colony is converted into honey (20 wt% water), the concentration of a pesticide in honey (A') becomes 1.6 times as high as that in sugar syrup (A).

little organophosphates remained in residual honey though some neonicotinoids remained in it. Judging from the fact that every experimental colony take enough amount of a pesticide, these findings suggest the followings: Organophosphates in honey stored in combs will be degraded in a short period of time and can become harmless to a honeybee colony soon; on the other hand, neonicotinoids will be persistent over a long period of time and can continue to adversely affect a honeybee colony through toxic honey stored in cells during overwintering. This suggest that a honeybee colony taking a neonicotinoid make it difficult to overwinter as previously discussed by Yamada et al. (2018b).

We calculated the ratio of the concentration of a pesticide in residual honey (B) to that in sugar syrup fed to a honeybee colony (A') after adjusting the water content in sugar syrup (50%) to that in honey (20%) as follows: B/A' = 0.141 (approximately, 7.09-fold dilution) for dinotefuran, 0.234 (approximately, 4.27-fold dilution) for clothianidin, 0.006 for fenitrothion and 0.000 for malathion, respectively as shown in Table 8. Neonicotinoids (dinotefuran and clothianidin) included in honey stored in cells seem to be hardly decomposed within a few months under the conditions of atmospheric temperatures (not so high), cells in a dark beehive, low water content in honey (ca. 20%), but organophosphates (fenitrothion and malathion) seem to be easily decomposed within a few weeks. Considering the fact in this work that every colony entered the overwintering on December 1st 2013 and honey in the neonicotinoid colonies (DF and CN) was sampled from cells on combs on January 5th 2014 when the colonies became extinct, the neonicotinoids seem to have been hardly decomposed and maintained in toxicity after a month in overwintering.

On the other hand, no or little organophosphate remained in honey in cells. Based on the findings that the neonicotinoid concentrations in sugar syrup (honey) were diluted about 4.27 to 7.09 times at the colony extinction in this work and no or little organophosphates could be found in residual honey, we can understand that the concentration of a pesticide administered to a bee colony in a field experiment is reduced by nectar from a controlled field of pesticide-free flowers and organophosphates are much more rapidly degraded than neonicotinoids.

DISCUSSION

Differences in the influence on a bee colony between neonicotinoids and organophosphates

Comparing the characteristics of neonicotinoids with those of organophosphates, organophosphates is extremely more rapidly degradable than neonicotinoids where there is a difference in persistency between a few days for an organophosphate (PPDB, 2017a, b) and a few years for a neonicotinoid (Hopwood, 2016; PPDB, 2016b). The long-persistency which may bring such benefits as a reduction in the load on farmers must cause serious disadvantages such as environmental pollution (disruption) and ecological disaster.

The advent of neonicotinoids with extremely-long persistency has heretofore resulted in unthinkable problems such as a winter loss of bee-colonies for bee-keeping (Seitz et al., 2016; van der Zee et al., 2015). From the difference in toxic persistence between neonicotinoids and organophosphates, we can deduce that a honeybee colony composed of honeybees which continue to ingest poisonous nectar, pollen and water from contaminated fields by long-persistent neonicotinoid are weakened before winter and the weakened colony eventually becomes extinct in winter when honeybees continue to take only poisonous food (honey and pollen) stored in cells on combs. On the other hand, as extremely short-persistent organophosphates are promptly lost in their toxicity the food stored in cells on combs is already non-poisonous in winter and a honeybee colony composed of honeybees which have taken organophosphates can probably succeed in overwintering. We infer that the increase in a massive colony loss in winter can be ascribed to a long-persistent pesticide such as a neonicotinoid.

Why did one of two control colonies and organophosphate colonies fail to overwinter?

The first thing that comes to mind as the cause of overwintering failure is a weak colony before winter. Examining the number of adult bees just before overwintering in each colony, they were 2189 in CR-1 (extinction during overwintering), 2904 in CR-2 (success in overwintering), 795 in DF (extinction), 867 in CN (extinction), 2213 in FT (extinction) and 1745 in MT (extinction) on December 1st 2013. As adult bees in the neonicotinoid colonies (DF and CN) were lesser than those in the other colonies (CR-1, CR-2, FT and MT) which were about on the same-level and the sooner extinction after turning in overwintering of the neonicotinoid colonies seems to be caused by weakening of the colony activity due to the long-persistent and strong toxicity of a neonicotinoid before overwintering, and furthermore, by the ingestion of prolonged toxic food stored in beehives due to the long-term persistency of the neonicotinoid during overwintering.

On the other hand, a change in the state of the organophosphate colonies (FT and MT) during overwintering cannot be explained by the same causes as the neonicotinoid colonies because the organophosphate colonies had about the same number of adult bees just before overwintering as the control colonies (CR-1 and CR-2). We suspect some conceivable causes on the extinction of the organophosphate colonies that a break of a bee-cluster was induced by the opening of the beehive to observe the inside and to take photographs during overwintering under cold climate conditions; this break was then proceeded by the wide fluctuations in atmospheric temperature after the beehive-opening and especially by sudden rise in temperature (Figure 7).

We experienced that even the colony to which higher concentration of fenitrothion was administered succeeded in overwintering as in the case of the control colony in the previous field experiment (Yamada et al., 2018b). Here, we will closely examine the difference of experimental results between previous work (Yamada et al., 2018b) and this work. The numbers of adult bees just before overwintering in the previous work conducted on December 13th 2012 were 7080 in the fenitrothion colony which succeeded in overwintering and 12,858 in the control colony which succeeded in overwintering, respectively. On the other hand, those in this work conducted on December 1st 2013 were 2,213 in the fenitrothion colony which failed in overwintering, 2,189 and 2,904 in the control-1 colony which failed in overwintering and the control-2 colony

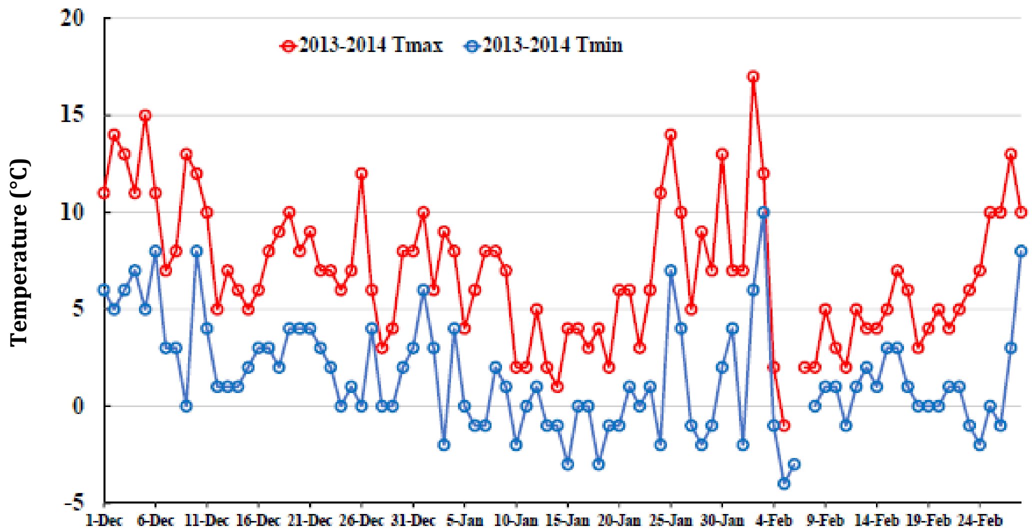

Figure 7: Temperature around the experimental site in overwintering from December 1st in 2013 to February 28th in 2014.

which succeeded in overwintering, respectively. Judging from the facts earlier mentioned, the number of adult bees in this work may probably be marginal number indicating whether a honeybee colony can succeed in overwintering or not. The earlier and more frequent openings of a beehive in this work during overwintering before March than the previous work can probably have weakened a honeybee colony because the opening of a beehive to take photographs was conducted only once on February 1st 2013 in the previous work but in this work the opening was twice on January 5th and February 7th 2014.

Here, we considered the possibility that a sharp change in temperature and a difference between the maximum and minimum temperatures in the day weakens the honeybee colony. Honeybees make a cluster in a beehive to protect them against the cold of winter. A cluster is sometimes broken in winter when a beehive is opened or the temperature is comparatively high. As the broken cluster is hard to form again soon, it is severely damaged by the coming cold. Figure 7 shows daily changes in temperature from December 1st to February 28th in the region of the experimental site in this work (2013 to 2014). It can be seen from Figure 7 that the temperatures around January 25th and February 2nd in this work suddenly changed and increased. This rise in temperature can probably cause a cluster break.

Only two neonicotinoid colonies (DF and CN) had already become extinct in all colonies at the first inspection of a beehive on January 5th 2014 in this work. The colony (CR-1, FT and MT) extinction after the first inspection seems to be caused by the plausible scenario that a weak colony composed of marginal number whether a honeybee colony can succeed in overwintering or not was damaged by the break of the cluster due to the first inspection and was further damaged by the subsequent first sudden change and rise in temperature around January 25th 2014, and after that, CR-1 and MT had become extinct on February 7th 2014, and FT also later became extinct after the additional damage due to the second sudden change and rise in temperature in February 2nd 2014. Eventually, only CR-2 which has the most adult bees of all colonies just before overwintering survived after overcoming the damage.

The uncertainty of field experiment (difficulties in controlling circumstances in a field experiment and difference in pesticide-concentration between a set value in administration to a colony and an actual value in ingestion by honeybees)

It is important in a field experiment to get plausible results that occur in a practical apiary though some differences between the natural environmental conditions and the experimental conditions may be offset by a control, but it is impossible to accurately reproduce the complicated natural surroundings of the experimental site.

There are the following difficulties to be solved in order to make the environment of a field experiment on a honeybee colony as close as possible like the environment of a practical apiary or elucidate the phenomena occurring in a practical apiary as accurately as possible by conducting field experiments: First, the initial number and age of members which divide the labor among honeybees are the same among every colony at the start of a field experiment. Second, the ovipositional ability of the queen is the same in every colony. Third, the initial numbers of brood and eggs in a honeybee colony are the same in every colony. Fourth, the initial amount of food (honey and pollen) stored in a beehive is the same in every colony. Fifth, the influences of a pesticide on a bee colony from the natural surroundings around the experimental site can be negligible though the influences of the weather on a bee colony seem to be offset by control colonies. Sixth, the influence of the beehive position can be negligible though a difference of the influence between the south and the north seems to be offset by setting two controls at both the southern and northern ends. Seventh, few predators can be found around the experimental site.

We tried to approximately equalize the initial conditions of each colony in order to solve the difficulties earlier mentioned. The initial numbers of adult bees and capped brood in each colony were adjusted to be on approximately the same level in every colony after the acclimatization period of about 9 weeks before the start of the experiment. The initial amount of food (honey and pollen) stored in each colony was also adjusted to be very roughly the same in every colony after about nine- week acclimatization period. The ovipositional ability of a queen cannot always be the same in every colony because the queen purchased from a bee-keeper was used without the minute examination of her ovipositional ability. The experimental site with no aerial crop dusting was selected. Both the pesticide-free watering place and flowering place were prepared in the immediate neighborhood of the experimental site. The experimental site was selected around where bears could not be found. Two control colonies were placed at both the southern and northern ends to offset the influence of the beehive position. The attacks of Japanese giant hornets (*Vespa mandarinia japonica*) could not entirely be prevented though various possible measures were taken. It is undeniable that the measures taken in this field experiment were inadequate as a result of cost and technical barriers.

Conclusion

A long-term field experiment was conducted over 241 days from August 13th 2013 to April 11th 2014. The pesticide concentrations in this work performed on the assumption of crop-dusting in the distance were only one-tenth of those in our previous work (Yamada et al., 2018b) performed on the assumption of crop-dusting in the neighborhood. The numbers of adult bees in the neonicotinoid colonies (dinotefuran and clothianidin) were less than one-half of those in other colonies just before overwintering. Both of the neonicotinoid colonies were the first to become extinct during overwintering. One of two control colonies (CR-1) and the malathion colony (organophosphate) became extinct about one month after the extinction of the neonicotinoid colonies. The fenitrothion colony (organophosphate) also became extinct about one month after the extinction of the malathion colony. Only one control colony (CR-2) which had the largest number of adult bees among all colonies just before overwintering succeeded in overwintering. We inferred from the circumstances of the control colony (CR-1) and organophosphate (fenitrothion and malathion) colonies which became extinct that the extinctions of organophosphate colonies which may possibly be caused by a cluster break in overwintering are different from the extinctions of neonicotinoid colonies (dinotefuran and clotjianidin) which can be probably caused by the toxicity of each neonicotinod pesticide ingested.

The aforementioned findings suggest that neonicotinoids which are long-persistent easily weaken a bee colony and make it difficult for the colony to overwinter but organophosphates which have very short-term effectiveness can rapidly lose their effectiveness and can hardly harmfully affect a bee colony during overwintering. All of the colonies had no mites and only two wax worms in the experiment.

From the analysis of residual pesticide in honey stored in an extinct colony, the following findings were obtained. Organophosphates (fenitrothion and malathion) could hardly be detected in honey stored in a honeybee colony. This fact suggests that they have been degraded in honey stored and can hardly cause a massive colony loss in winter. Neonicotinoids which were at lower concentration in residual honey in an extinct colony than in sugar syrup (converted into honey) was administered to a honeybee colony; the concentration of dinotefuran detected in residual honey was about one-seventh of the concentration in sugar syrup administered to the DF, and the concentration of clothianidin detected in residual honey was about one-third of the concentration in sugar syrup administered to the CN. These facts suggest that a neonicotinoid maintains its toxicity during overwintering and continues to harmfully affect a honeybee colony over a prolonged period even during overwintering and can also accelerate the colony extinction. From a high LD_{50} value (time required for 50% dissipation of the initial concentration) of a neonicotinoid whose toxicity is occasionally effective for a few years, we can infer that the dilution of the neonicotinoids in honey stored can be hardly caused by their degradation but can result from being diluted by pesticide-free flowers and water. As previously described in the study of Yamada et al. (2012, 2018a, b), such dilution can be easily considered in a practical apiary.

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REFERENCES

Alkassab AT, Kirchner WH (2016). Impacts of chronic sublethal exposure to clothianidin on winter honeybees. Ecotoxicology. 25(5):1000-1010. doi:10.1007/s10646-016-1657-3. http://link.springer.com/article/10.1007/s10646-016-1657-3.

Durkin PR (2009). Dinotefuran-human health and ecological risk assessment-final report. SERA (Syracuse Environmental Research Associates, Inc.), TR-052-18-03b. http://www.fs.fed.us/foresthealth/pesticide/pdfs/0521803b_Dinotefuran.pdf.

Hodgson E (2012). Insecticidal Seed Treatments can Harm Honey Bees.ICM News, April 6, 2012, Integrated Crop Management (ICM), Iowa State University (ISU) Extension and Outreach. http://crops.extension.iastate.edu/cropnews/2012/04/insecticidal-seed-treatments-can-harm-honey-bees.

Hopwood J, Code A, Vaughan M, Biddinger D, Shepherd M, Black S-H, Lee-Mäder E, Mazzacano C (2016). How Neonicotinoids Can Kill Bees -The Science Behind the Role These Insecticides Play in Harming Bees, 2nd Edition; Revised & Expanded-.The Xerces Society for Invertebrate Conservation, Portland, OR, USA. http://ento.psu.edu/publications/are-neonicotinoids-killing-bees.

Iwasa T, Motoyama N, Ambrose JT, Roe RM (2004). Mechanism for the differential toxicity of neonicotinoid insecticides in the honey bee. Crop Protect. 23:371-378. http://www.sciencedirect.com/science/article/pii/S0261219403002308.

Kakuta H, Gen M, Kamimoto Y, Horikawa Y (2011). Honeybee exposure to clothianidin: analysis of agrochemicals using surface enhanced Raman spectroscopy. Res. Bull. Obihiro Univ. 32:31-36. http://jglobal.jst.go.jp/public/20090422/201102260879015920.

Laurino D, Manino A, Patetta A, Porporato M (2013). Toxicity of neonicotinoid insecticides on different honey bee genotypes. Bull. Insectol. 66 (1):119-126. ISSN: 1721-8861. http://www.bulletinofinsectology.org/pdfarticles/vol66-2013-119-126laurino.pdf#search=%27Toxicity+of+neonicotinoid+insecticides+on+different+honey+bee+genotypes%27.

Lu C, Warchol KM, Callahan RA (2012). *In situ* replication of honeybee colony collapse disorder. Bull. Insectol. 65(1):99-106. http://www.bulletinofinsectology.org/pdfarticles/vol65-2012-099-106lu.pdf.

Lu C, Warchol KM, Callahan RA (2014). Sub-lethal exposure to neonicotinoids impaired honey bees winterization before proceeding to colony collapse disorder. Bull. Insectol. 67(1):125-130. http://www.bulletinofinsectology.org/pdfarticles/vol67-2014-125-130lu.pdf.

Nakamura J, Kimura K, Takahashi J, Sasaki M, Manabe N (2014). About the issues on Yamada's paper on neonicotinoid pesticides. Chem. Today. 514:67-68.

NUFARMNZ (2012). Safety Data Sheet Product: Name: CATERKIL1000: Infosafe No™ 3NUDE. NUFARM in New Zealand (NUFARMNZ), August 2012, Issued by NUFARMNZ. http://www.nufarm.co.nz/assets/18373/1/Caterkil_1000_SDS.pdf.

Pesticide Properties Data Base (PPDB) (2016a). "Dinotefuran" (Ref: MTI 446). University of Hertfordshire.http://sitem.herts.ac.uk/aeru/ppdb/en/Reports/1195.htm.

Pesticide Properties Data Base (PPDB) (2016b). "Clothianidin" (Ref: CGA 322704). University of Hertfordshire. http://sitem.herts.ac.uk/aeru/ppdb/en/Reports/171.htm.

Pesticide Properties Data Base (PPDB) (2017a). "Fenitrothion" (Ref: OMS 43). University of Hertfordshire.http://sitem.herts.ac.uk/aeru/ppdb/en/Reports/299.htm.

Pesticide Properties Data Base (PPDB) (2017b). "Malathion" (Ref: OMS 1). University of Hertfordshire.http://sitem.herts.ac.uk/aeru/ppdb/en/Reports/421.htm.

Sanford MT (2003). Protecting honey bees from pesticides. A series of the Entomology and Nematology Department, Florida Cooperative Extension Service, Institute of Food and Agricultural Sciences, University of Florida, CIR534. http://pesticidestewardship.org/PollinatorProtection/Documents/AA14500.pdf.

Seitz N, Traynor KS, Steinhauer N, Rennich K, Wilson ME, Ellis JD, Rose R, Tarpy DR, Sagili RR, Caron DM, Delaplane KS, Rangel J, Lee K, Baylis K, Wilkes JT, Skinner JA, Pettis JS, van Engelsdorp D (2016). A national survey of managed honey bee 2014–2015 annual colony losses in the USA. J. Apicult. Res. 54(4):292-303. http://dx.doi.org/10.1080/00218839.2016.1153294.

Stevenson JH (1978). The acute toxicity of unformulated pesticides to worker honey bees (*Apis mellifera L.*). Plant Pathol. 27:38–40. http://onlinelibrary.wiley.com/doi/10.1111/j.1365-3059.1978.tb01070.x/full.

The Japan Food Chemical Research Foundation (JFCRF) (2017). Maximum Residue Limits (MRLs) of Agricultural Chemicals in Foods: Compositional Specification for Foods (Updated on July 19, 2017). The Japanese Positive List System for Agricultural Chemical Residues in Foods (Document released by Ministry of Health, Labour and Welfare). http://www.ffcr.or.jp/zaidan/FFCRHOME.nsf/pages/MRLs-p.

United States Environmental Protection Agency (U.S.EPA) (2004). Pesticide Fact Sheet, Name of Chemical: Dinotefuran.http://www.epa.gov/opp00001/chem_search/reg_actions/registration/fs_PC-044312_01-Sep-04.pdf#search='Pesticide+Fact+Sheet%2C+Name+of+Chemical%3A+Dinotefuran.

United States Environmental Protection Agency (U.S.EPA) (2004). Pesticide Fact Sheet, Name of chemical: Dinotefuran Reason for Issuance: Conditional Registration Year Issued: September 2004. Office of Prevention, Pesticides and Toxic Substances (7501C).http://www.epa.gov/opp00001/chem_search/reg_actions/registration/fs_PC-044312_01-Sep-04.pdf#search='Pesticide+Fact+Sheet%2C+Name+of+Chemical%3A+Dinotefuran.

van der Zee R, Gray A, Pisa L, de Rijk T (2015). An observational study of honey bee colony winter losses and their association with varroa destructor, neonicotinoids and other risk factors. PLoS ONE 10(7):e0131611. Doi: 10.1371/journal. http://journals.plos.org/plosone/article?id=10.1371/journal.pone.0131611.

Wang D, Naito H, Nakajima T (2012). The toxicity of fenitrothion and permethrin. Insecticides - Pest Engineering, Dr. Farzana Perveen (Ed.), InTech. ISBN: 978-953-307-895-3.http://www.intechopen.com/books/insecticides-pest-engineering/the-toxicity-of-fenitrothion-and-permethrin.

World Health Organization (WHO) (2010). WHO Specifications and evaluations for public health pesticides: Fenitrothion: *O,O*-dimethyl *O*-4-nitro-*m*-tolyl phosphorothioate. http://www.who.int/whopes/quality/Fenitrothion_specs_eval_WHO_Jan_2010_ok.pdf?ua=1.

Yamada T, Yamada K, Wada N (2012). Influence of dinotefuran & clothianidin on a bee colony. Japanese J. Clin. Ecol. 21(1):10-23. http://dspace.lib.kanazawa-u.ac.jp/dspace/bitstream/2297/37606/1/SC-PR-YAMADA-T-10.pdf.

Yamada T, Yamada K, Yamada Y (2018a).A clear difference in the impact on a honeybee (*Apis mellifera*) colony between the two vehicles of sugar syrup and pollen paste. J. Biol. Ser. 1(3):084-107. doi: 10.15413/jbs.2018.0400.

Yamada T, Yamada Y, Yamada K (2018b). Difference between the impact of the neonicotinoid dinotefuran and organophosphate fenitrothion on a bee colony in a long-term field experiment: An evidence. J. Biol. Ser. 1(3):108-137. doi: 10.15413/jbs.2018.0401.

Yokoyama S, Ito M, Nagasawa S, Morohashi M, Ohno M, Todate Y, Kose T, Kawata K (2015). Runoff and degradation of aerially applied dinotefuran in paddy fields and river. Bull. Environ. Contam. Toxicol. 94:796–800. doi: 10.1007/s00128-015-1554-0. http://www.ncbi.nlm.nih.gov/pubmed/25917847.