

Seasonal Changes in the Size and Mite-Prevalence of A Bee Colony Exposed to Dinotefuran via Pollen Paste and Damaged by *Varroa* Mites

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Simple Summary

In this study, the impact of dinotefuran (DF) administered via pollen paste to a bee colony damaged by *Varroa* mites was investigated. Seasonal changes in the number of adult bees (those capped brood) and the mite-prevalence among adult bees were measured through a field experiment over 180 days. It was found that the bee colony collapsed under the intake of a smaller amount of DF due to the synergistic effect of DF and mite-damage. Because the daily pesticide-free sugar-syrup intake per bee in the DF-exposed colony administered via pollen paste was greater than that of the control colony, DF may have an appetite-promoting effect. Since the consumption of DF-containing pollen paste per bee per day showed almost no difference between all colonies, DF seems to have no repellent effect. Mite-prevalence continued to rise and became almost 100% near the time of colony extinction. The inner-temperature-fluctuation-range of the hive-box (T_i) was smaller than that of the ambient temperature (T_a). The inner-temperature-fluctuation-range of the DF-exposed-colony hive-box was larger than that of the control-colony hive-box. If T_a was 30°C or less, T_i became higher than T_a ; if T_a was 30°C or more, T_i became lower than T_a .

Abstract

Neonicotinoids, such as dinotefuran (DF), have caused a variety of problems, such as massive loss and winter failure of bee colonies as the price for reducing farm work, as such neonicotinoids continue to maintain high insecticide activity over a long period of time. In this study, a field experiment was conducted over about six months to investigate the effects of DF on bee colonies damaged by *Varroa* mites. This study examined the long-term changes in the sizes of bee colonies, the intake of sugar syrup (SS), the intake of pollen paste (PP) (which is a vehicle for administering DF), the intake of DF, the mite-prevalence of bees, and the inside and outside temperatures of the hive-boxes. The variation width of the inner temperature of the hive-box was less than that of the ambient temperature (T_a). The inner temperature of the hive-box was adjusted to about 30°C of T_a as the boundary. If T_a was lower than 30°C, the inner temperature of the box was higher than T_a , and if T_a was higher than 30°C, the inner temperature was lower than T_a . The temperature variation width of the DF-exposed colony was greater than that of the control colony. The average intake of SS per bee per day in the DF-exposed colony was greater than that of the control colony. The average intake of PP per bee per day in the DF-exposed colony was almost equal to that of the control colony. These results suggest that bees do not avoid DF and ingest PP without making a distinction between toxic and pesticide-free PP. In the period from the start of DF administration to colony extinction, the intake of DF per colony was about 865 µg/colony, the intake per bee was 14 ng/bee, and the intake per bee per day was less than 0.1 ng/bee/day. These intakes are much lower than the previous ones recorded (60–65 ng/bee, 0.27–2.32 ng/bee/day). These discrepancies may be because attacks from mites and Japanese giant hornets hastened the colony's collapse. Seasonal changes in the mite-prevalence of honeybees were approximately the same regardless of the bee colonies. At the end of August (the start of the attacks by Japanese giant hornets), the mite-prevalence increased rapidly. Even if the number of bees damaged by mites decreased, the mite-prevalence continued to increase, approaching 100% before the bee colonies became extinct. In this study, it was found that bee colonies collapse via the intake of a smaller amount of DF due to the synergistic effect of DF and mite-damage. To prevent a bee colony collapse, beyond minimizing the adverse effects to a bee colony from neonicotinoids such as DF with long-term residual effects and high insecticide properties, it is necessary to reduce the damage from mites as much as possible while considering the synergistically adverse effects of neonicotinoids and miticides.

Keywords: Seasonal change; Honeybee; Adult bee; Capped brood; *Varroa* mite; Colony size; Dinotefuran; Neonicotinoid; Mite-prevalence; Field experiment

Introduction

Neonicotinoid pesticides, which were invented at the end of the 20th century, are strongly suspected to be the cause of the large-scale bee colony loss that has occurred in the 21st century, based on laboratory-level experiments. Some scientists, however, have argued that laboratory-level experiments do not necessarily reproduce the phenomena that occur in actual beekeeping. To dispel this doubt, using an environment that approximates an actual beekeeping location, long-term field experiments have revealed the following issues related to honeybee colonies: the possibility that neonicotinoid pesticides can cause colony collapse disorder (CCD) [1-3]; the differences in the impact between sugar syrup (SS) and pollen paste (PP) containing neonicotinoid pesticides [4]; the differences in the impact between neonicotinoid pesticides and organophosphorus pesticides on honeybee colonies [5,6]; the differences in the impact of pesticides between a mite-existing region (Japan) and a mite-free region (Maui) [7]; the differences in the impact of pesticides between a region with clear seasonal changes (Japan) and a region with no seasonal changes (Maui) [7,8]; and the impact of neonicotinoid pesticides on the winterization of honeybee colonies [9].

According to the field experiment, all neonicotinoid-exposed colonies became extinct (100% extinction rate), but the extinction rates of the control colony and the organophosphate-exposed colony were about the same and much lower than the rates of the neonicotinoid-exposed colony [4,5,7]. From these field experiments, neonicotinoid pesticides, which have strong insecticidal activity and long-term residual effects, are presumed to cause serious damage to bee colonies and sometimes collapse them.

It is known that not only neonicotinoid pesticides but also external parasitic mites such as *Varroa* mites can greatly reduce the activity of honeybee colonies. So far, there have been many reports on damage to honeybee colonies by pests such as *Varroa* mites, wax-moth larvae, and small hive beetles.

There have been many reports on the relevant effects of the ectoparasitic mite *Varroa destructor* and the deformed wing virus (DWV) closely associated with the mite, which can seriously damage honeybee colonies and sometimes collapse them. Indeed, the impact of *Varroa* mites on honeybee colonies have been investigated around the world [10], including in Switzerland [11], southeastern Brazil [12], South Africa [13], Mexico [14], southern Spain [15], and northern Iraq [16].

The cause of the massive loss and overwintering failure of bee colonies that occurred in the world's apiaries in the early 21st century was explored, and it is now presumed that *Varroa* mites (DWV) are one of the causes of this loss of colonies [17-20].

There have also been reports on *Varroa*-mite-damage prevention measures [21-27]. The damage to bee colonies by pests other than the *Varroa* mite was investigated, and countermeasures against these pests, such as the small hive beetle [28-34] and wax moth larva [35-40], were reported.

Furthermore, research into bee viruses that can weaken honeybee colonies or occasionally collapse them has been carried out. To understand the global infection status of bee viruses, an extensive literature survey on the geographic distribution of major viruses (DWV, acute bee paralysis virus, sacbrood virus, etc.) infecting *Apis mellifera* bees was conducted, and their temporal population dynamics were reviewed [41].

Field experiments were also conducted in a *Varroa*-mite-free area to counter the theory that the cause of the massive loss and wintering failures of honeybee colonies was not neonicotinoid pesticides but mites. Experimental results in Maui and Australia without *Varroa* mites demonstrated that the massive loss of honeybee colonies is caused by long-term residual pesticides with high toxicity, such as neonicotinoid pesticides, rather than *Varroa* mites [7,8,42]. These results suggest that neonicotinoid pesticides are one of the main causes of the massive loss of honeybee colonies.

We previously revealed through long-term field experiments that the neonicotinoid pesticides dinotefuran and clothianidin have a serious adverse effect on bee colonies [1,4-8]. However, the effects of neonicotinoid pesticides on bee colonies damaged by *Varroa* mites have not been clarified during field experiments. This study examines the effects of the neonicotinoid pesticide dinotefuran (DF) on honeybee *Apis mellifera* colonies damaged by *Varroa* mites (which have not been reported so far) through long-term field experiments.

Materials and Methods

Materials and Preparation of Pesticide Concentrations

Starkle Mate® (10% DF; Mitsui Chemicals Aglo, Inc., Tokyo, Japan) was used in this study.

Preparation of SS

Granulated sugar was purchased from the Japan Beekeeping Association (<http://www.beekeeping.or.jp/>) and was composed of 99.7988% purified sugar (granulated sugar), Sodium chloride (salt) 0.1% or more, L-lysine hydrochloride 0.1% or more, and food dye (Blue No. 2) 0.0012% or more. A total of 20 kg of granulated sugar and 13.33 kg of hot water at about 75°C were mixed in a 50 L plastic tank, and then 60% SS of granulated sugar was produced.

Preparation of SS Containing DF for Toxic PP

1 kg of 100 ppm-DF-SS was produced using SS containing 60 wt% of sugar and Starkle Mate® (10% DF; Mitsui Chemicals Aglo, Inc., Tokyo, Japan). The prepared SS containing DF in a 10 L-container was blocked from light using a black bag and stored in a refrigerator.

Preparation of PP without DF

In total, 25 kg of pollen from Spain was purchased from Tawara Apiaries Co., Ltd., Kobe, Japan (<https://tawara88.com/about.html>). The pollen was used after it was lightly ground with “Kona Ace A-7” flour milling equipment manufactured by Kokkousha Co., Ltd., Nagoya, Japan (<http://www.kokkousha.co.jp/>). The viscosity of PP was determined by the ratio of SS in PP, the particle size of the pollen, and the temperature of the PP. The pollen and SS that did not fall when the PP-filled tray was turned upside down was preliminarily examined. As a result, 60% pollen by weight and 40% SS by weight was confirmed to be an appropriate ratio. Pesticide-free and PP containing 0.4 ppm of DF were prepared in 10 kg. Pesticide-free PP (6 kg of pollen and 4 kg of SS (pesticide-free)) was prepared in a large plastic bucket by kneading with a drill screwdriver with stirring blades used for high viscosity at a low speed until achieving a uniform paste.

Preparation of PP without DF

PP containing dinotefuran was prepared in the same manner used for pesticide-free PP. By adding 40 g of 100 ppm-DF-SS into 3960 g of pesticide-free water, 4 kg of 1 ppm-DF-SS was prepared, containing 100 ppm-DF-SS and 1 ppm-DF-SS mean sugar syrup containing 100 ppm and 1 ppm of dinotefuran, respectively; 10 kg of 0.4 ppm-DF-PP was prepared by kneading 6 kg of pollen and 4 kg of 1-ppm-DF-SS, where the 0.4 ppm-DF-PP contained pollen paste with 0.4 ppm of dinotefuran. The DF concentration of the PP in this work (0.4 ppm-DF) was set to about 70% of the dinotefuran concentration (0.565 ppm in PP) used in the 2011/2012 experiments [4].

Preparation and maintenance of the tray filled with PP

The 300 g PP, after being weighed in an upper plate balance scale (accuracy±1 g), was packed into a foamed polystyrene tray. Then, the PP tray filled with pollen paste was wrapped to prevent the evaporation of moisture from the tray and stored in a refrigerator to prevent alteration of the PP. The pesticide-free PP tray was stored in the refrigerator compartment, and the PP tray containing DF was stored in the freezer compartment.

Long-Term Field Experimental Method

Experimental Site and Arrangement of the hive-box

The experiment was conducted at a location (Latitude 37° 2' 35" N; Longitude 136° 45' 38" E; 70 m above sea level) with a house about 3.5 km away in the north-northwest direction from the previous experimental site [4-7]. It was leveled in advance to serve as a weeded and flat experimental site. Six hive-boxes were used for the experiments, with entrances arranged at about 80 cm intervals in the south direction, three control colonies (CR-1, CR-2, CR-3) towards the west and the east, and three DF-exposed colonies (DF-1, DF-2, DF-3). Each hive-box was placed on a stand about 20 cm in height, and a large PVC tray (with several 3 mm perforations) protruding far from the entrance side of the hive-box in front was placed between the hive-box and the stand. The many holes in the tray were used to prevent water from accumulating in the tray for the measurement of the number of bee deaths.

Preparation for the Field Experiment

We numbered the inner walls and bottoms of the hive-boxes used in the experiment. At this stage, towards the front of the entrance, the numbers clockwise on each wall and the bottom was described as the "Bottom" (Supplementary Figure S1). A symbol was added on both sides of the comb frame from the left toward the front of each hive-box (Supplementary Figure S2) so that a photograph could be used to identify the object. The left and right sides of the comb frame toward the entrance of the hive-box were called "F" (front) and "B" (back). For example, "CR-2-3B" means the right side ("B") of the third comb frame ("3") in the second control-colony hive-box ("CR-2").

The experiment began on June 19, 2018 using eight hive-boxes consisting of four comb frames with honeybees. The state of each honeybee colony was observed until July 1, 2018 without pesticide administration. Six colonies that appeared to have similar conditions were selected from among the eight prepared colonies, and the experiments of pesticide (DF) administration began on July 1, 2018, where three colonies were DF-exposed colonies, and three colonies were pesticide-free (control) colonies. The remaining two colonies that were not used in the experiment were used as spares when an accident occurred in the experimental colony. All the queen bees in the eight colonies were connected. We began to conduct observational experiments about every two weeks starting from July 1, 2018. In the observational

experiment, the results were recorded by taking photographs of all the comb frames with and without honey bees and the hive-boxes with honeybees, as well as counting the number of dead bees. Furthermore, all honeybee colonies were fed 800 g of SS (pesticide-free); 300 g pesticide-free PP was fed into three control colonies (CR-1, CR-2, CR-3), and 300 g of PP containing 0.4 ppm of DF was fed into the three DF-exposed colonies (DF-1, DF-2, DF-3).

Before the start of the experiment from June 19 to July 1, the average intakes of SS and PP among honeybee colonies were measured to reduce the remaining amounts of SS and PP as much as possible. The amounts of SS and PP to be fed to each colony for every observational experiment were determined to be 800 g and 300 g, respectively. In addition, the remaining amounts of SS and PP were accurately measured with an upper plate balance and recorded each time for each observational experiment.

After controlling the feeding amounts of SS and PP, the remaining amounts were often zero. When there was a remaining amount (once), the PP containing 0.4 ppm of DF after the measurement was stored in a thick plastic bag. The remaining PP was 4912.6 g in total, which is equivalent to 2 mmg of DF. After completion of the experiment, a hole about 50 cm in diameter and about 50 cm in depth was dug between the trees in the central part of the site (about 825 m²) owned by Toshiro Yamada, the DF-containing PP was discarded in the hole, and the hole was filled. After the measurement, all the participants of the experiment checked the experimental status and recorded their observations. In addition, the experiment was conducted until mid-April when the whole bee colony collapsed or succeeded in overwintering.

Procedures and Instructions for the Field Experiment

The most important data are the number of adult bees and the number of capped brood. To obtain these data as accurately as possible, 1) the honeybee outside the hive-box was as small as possible. To achieve this, the experiment was started immediately after dawn (the starting time of the experiment was determined by referring to the sunrise time. The experiment started at 5:30 a.m. from July to September and at 6:00 a.m. from October onwards. 2) The comb frames with bees were pulled out one by one to take pictures and prevent the bees from flying away. To do so, I gently pulled out the comb frame and set the comb frame with bees into a photography stand (Supplementary Figure S3) that was made in advance to take a photo. 3) Bees may become restless due to wind and rain, so bad weather conditions should be avoided. Using the weather forecast, the experiment was carried out to avoid bad weather as much as possible. On the day, if the weather suddenly worsened, the experimental date was changed. Considering the above precautions, a field experiment with uncontrollable factors was performed using the procedure shown in Supplementary Method S1 to obtain the experimental data as accurately and correctly as possible.

How to Count the Number of Bees, the number of Capped Brood, and the Number of Bees Damaged by Mites

Using the photographs taken as described above, it was possible to determine the number of adult bees, the number of capped brood, and the number of mite-damaged bees. Counting these numbers from photographs is an extremely difficult task. Therefore, with the cooperation of Mr. Yoshiaki Nagai of Nanosystem Co., Ltd., Kyoto, Japan (<http://nanosystem.jp/firm.htm>) (which develops image processing software), I developed computer software in 2012 that automatically counts the relevant numbers from photographic images,

and improvements have since been made to the software. The accuracy and operation of the count have been greatly improved. The count time and count accuracy using this software are significantly improved compared to the amount of work required to count by hand directly from the original photo.

However, these counts are still inaccurate because of bee overlap, out-of-focus images, extreme contrast differences in the images, misjudgments of the counting targets, and so on. In this software, it is also possible to manually correct after automatic counting. Therefore, after automatic counting, the counting mistakes due to the automation were corrected by manual operation while zooming into the image, and the corrected number became the final data. Thus, errors still emerge in automation when using this software, but by using manual operation, it is possible to correct the count errors. The improvements in the counting accuracy and speed under this software contributed greatly to the improvement of the data analysis accuracy and the speed in our long-term field experiments. An overview of this automatic counting system is provided below.

Software Developed to Assist in Accurately Counting the Numbers of Adult Bees, Capped Brood, and Mite-Damaged Bees

This software was developed to count the numbers of adult bees and capped brood accurately. The following explains how to count the number of adult bees present in the image of a comb frame and how to count the number of capped brood using the image read. These numbers are counted with software using the image from a photo imported into the computer. The number of adult bees in the colony is obtained by summing the numbers of adult bees on both sides of all the comb frames with the bees and the number of remaining adult bees in the hive-box without the comb frame. The number of capped brood in a colony can be obtained by summing the numbers of capped brood on both sides of all the comb frames without bees. The number of adult bees damaged by the *Varroa* mite can be obtained from the same photo used to count the adult bees. How to count bees damaged by *Varroa* mites is described in a separate section. The measurement results obtained by this method, as long as the image remains, can be verified by anyone at any time. Here, the determination procedure of these numbers (limited to the number of adult bees and capped brood of the comb frame) are described in Supplementary Method S2.

Counting Method for the Number of Adult Bees Damaged by *Varroa* Mites

Various sampling methods for *Varroa* mites in a honeybee colony, such as the sticky board method, the roll method, and the powder sugar shake method, have been reported [43-45]. With these methods, it is impossible to determine the total number of *Varroa* mites in a bee colony.

It is presumed that the impacts of *Varroa* mites on a bee colony appear directly on the adult bees with damage caused by the mites. This paper aims to measure the total number of bees damaged by mites, not the total number of mites in the bee colony. To count the number of mite-damaged bees as accurately as possible, the images and software used to count the number of adult bees were used again. Using this counting technique for the number of adult bees, it is relatively easy to count *Varroa* mites on an adult bee. On the other hand, it is very difficult to count *Varroa* mites that causes damage to a brood in a comb cell. If any damage is received from *Varroa* mites during the brood stage, there should be a trace of the damage even if the brood becomes an adult bee. An adult bee with traces of its brood stage is considered to be a mite-damaged bee. Moreover, it is also conceivable that the mite dropped out

of the adult bee during the bee's development. In this case, it was confirmed whether mite traces remain in the adult bee. Therefore, an adult bee with traces of a mite falling off are also regarded as a mite-damaged bee.

The criteria for the determination of such traces were created based on the information obtained by expanding the image (Supplementary Method S3). The examples certified to be mite-damaged bees are shown in Figure 1. According to this criterion, using the photographic image taken for the adult number measurement, it is possible to evaluate the situation of the *Varroa*-mite damage among almost the total number of adult bees in the bee colony. Therefore, the total number of both adult bees with *Varroa* mites and those with traces of *Varroa*-mite damage was determined manually with the software used to measure the number of adult bees while enlarging the adult bee number measurement image. The measurement of the number of mite-damaged bees was a very difficult task and took considerable time (about 6 months). To eliminate the errors between operators, this measurement was carried out by only one person.

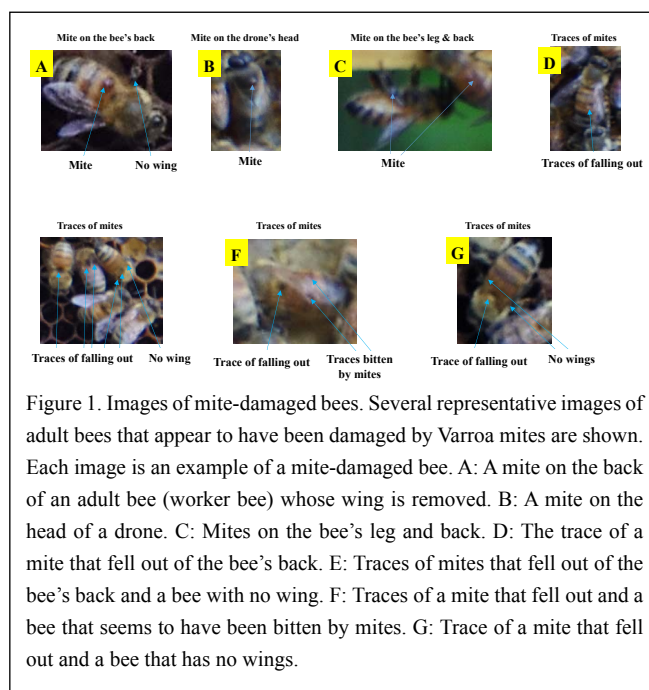


Figure 1. Images of mite-damaged bees. Several representative images of adult bees that appear to have been damaged by *Varroa* mites are shown. Each image is an example of a mite-damaged bee. A: A mite on the back of an adult bee (worker bee) whose wing is removed. B: A mite on the head of a drone. C: Mites on the bee's leg and back. D: The trace of a mite that fell out of the bee's back. E: Traces of mites that fell out of the bee's back and a bee with no wing. F: Traces of a mite that fell out and a bee that seems to have been bitten by mites. G: Trace of a mite that fell out and a bee that has no wings.

Results

The Period, Site, and Purpose of the Long-Term Field Experiment

Long-term field experiments have been conducted four times at an apiary owned by the author in Shika-machi, Hakui-gun, Ishikawa Prefecture, Japan, and once on Maui Island. These experimental conditions and the purpose of the experiment are described in Supplementary Table S1. The main points of these five experiments and this experiment are as follows: (1) 2011 experiment (Shika), "Impacts of neonicotinoid pesticides on bee colonies (related to CCD)"; (2) 2011/2012 experiment, (Shika) "Impacts of pesticide intake pathways (SS, PP) on bee colonies"; (3) 2012/2013 experiment, "Differences in the impacts of neonicotinoid pesticides on bee colonies between organophosphorus pesticides"; (4) 2013/2014 experiment, "Impacts of low concentrations of neonicotinoid and organophosphorus pesticides on bee colonies"; (5) 2013/2014 experiment, "Impacts of pesticides on bee colonies in areas without both mites and four seasons"; (6) 2018 experiment, "Reproduction experiment of the impact of PP with DF on bee colonies (one of 2011/2012 experimental results)" (this work).

Observation Results in the Field Experiment

Seasonal changes in the size (the numbers of adult bees and capped brood) and the mite-prevalence among adult bees were investigated in bee colonies damaged by *Varroa* mites through a long-term field experiment over 180 days, which was conducted as follows.

Three colonies of control (pesticide-free) (CR-1, CR-2, CR-3) and three dinotefuran-exposed colonies with a PP containing 0.4 ppm DF (DF-1, DF-2, DF-3) were placed from west to east every 80 cm, with the front of the hive-box facing south (Supplementary Figure S4). On June 19, 2018, pesticide-free PP (300 g) and pesticide-free SS (800 g) were administered into all 8 colonies, and the experiment was started. Pesticide-free SS was administered to all colonies over the entire period of the experiment. After that, the state of each hive-box was carefully observed, and the six bee colonies that seemed to have almost the same activity were selected. Using these six bee colonies, the pesticide administration experiment began in July, 2018.

From mid-August 2018, all bee colonies began to be attacked by Japanese giant hornets, which continued until early December in 2018. From the beginning of September to the end of October, these attacks were particularly intense. To prevent attacks from Japanese giant hornet, a hornet capturing device was attached to the front of each hive-box in mid-August. At the beginning of October, the presence of *Varroa* mites in a few bee colonies was visually confirmed by the changes in experimental conditions, and acaricide was not administered into the bee colonies. In mid-November, all the hive-boxes were covered with a 5 cm thick foamed-polystyrene plate to protect from the cold (preparation for overwintering). The weather on the experimental (measurement) date and that at the start time and the end time are summarized in Supplementary Table S2. The experiment (measurement) was started as early as possible, shortly after dawn, before the outer bees went out to forage.

The situation at the time of experiments is summarized in Supplementary Table S3 (control colonies) and Supplementary Table S4 (DF-exposed colony). These tables reveal the following: The queen bee in the DF-exposed colony left, while the queen bee in the control colony remained until the extinction of the colony: There was little damage due to wax-moth larvae in all colonies. All the colonies were expected to be included in overwintering, with the exception of one colony (CR-1) of the controls (which became extinct on December 16): All the remaining colonies became extinct before overwintering. DF-3 became extinct early on 23 October, and CR-2, CR-3, DF-1, and DF-2 became extinct on 2 December, just after the start of overwintering (the slowest was CR-1 on December 16). These extinctions are presumed to have been caused by the spread of mites in addition to the attacks on Japanese giant hornets. The bee colony that appeared to have high activity at the beginning of the experiment rapidly declined after the attacks from the Japanese giant hornets, and all bee colonies became extinct around the start of overwintering.

The Number of Adult Bees

The field experiment, which started on June 19 and had continued observations about every two weeks, was completed on December 16, about six months later (180 days). Based on the photographs that continued to be taken on every observation day, the number of adult bees, the number of capped brood, and the number of bees damaged by mites were determined. The number of adult bees and the number of capped brood obtained in this way are displayed together in Table 1. Further, the number of adult bees in the table is shown in Figure 2. The number of measurements of mite-damaged bees will be provided in the Discussion section.

Table 1 Numbers of adult bees and capped brood

Date	k	Δt_k [day]	t_k [day]	CR-1		CR-2		CR-3		DF-1		DF-2		DF-3		Remarks
				$a(t_k)$	$b(t_k)$	$a(t_k)$	$b(t_k)$	$a(t_k)$	$b(t_k)$	$a(t_k)$	$b(t_k)$	$a(t_k)$	$b(t_k)$	$a(t_k)$	$b(t_k)$	
19-Jun-18	1	0	0	8613	5341	8343	3065	6987	6861	9010	5891	9067	3241	7079	7726	Start of Experiment
1-Jul-18	2	12	12	10507	11056	8439	9401	9830	8621	10188	9341	8926	12549	10071	5374	Start of DF administration
16-Jul-18	3	15	27	13064	10657	12402	7447	12016	8060	12070	8360	13726	8755	10491	8032	
28-Jul-18	4	12	39	12439	5688	11658	7640	10037	7006	11239	5443	11252	5769	10301	9052	
12-Aug-18	5	15	54	6977	3817	9812	4619	6965	4078	8437	2777	8588	4030	9213	4840	
28-Aug-18	6	16	70	6284	4125	9902	6629	6583	5277	7609	466	7558	5690	5662	4504	
11-Sep-18	7	14	84	7369	4755	8589	5050	8081	6262	4716	395	8742	5928	4204	2978	Attacks by giant hornets
23-Sep-18	8	12	96	6427	4201	9102	5636	6891	6487	2370	200	8046	6559	1158	1266	Attacks by giant hornets
6-Oct-18	9	13	109	3680	1490	8593	5982	6150	5015	964	105	7599	6091	41	1228	Attacks by giant hornets
23-Oct-18	10	17	126	1187	658	6395	4200	3461	2773	309	70	4366	3113	0	1234	Attacks by giant hornets
4-Nov-18	11	12	138	596	440	3373	1747	1332	1043	204	43	1957	1646			Attacks by giant hornets
18-Nov-18	12	14	152	207	411	383	913	254	607	112	39	191	1147			
2-Dec-18	13	14	166	56	460	0	989	0	691	0	72	0	1291			
16-Dec-18	14	14	180	0	463											

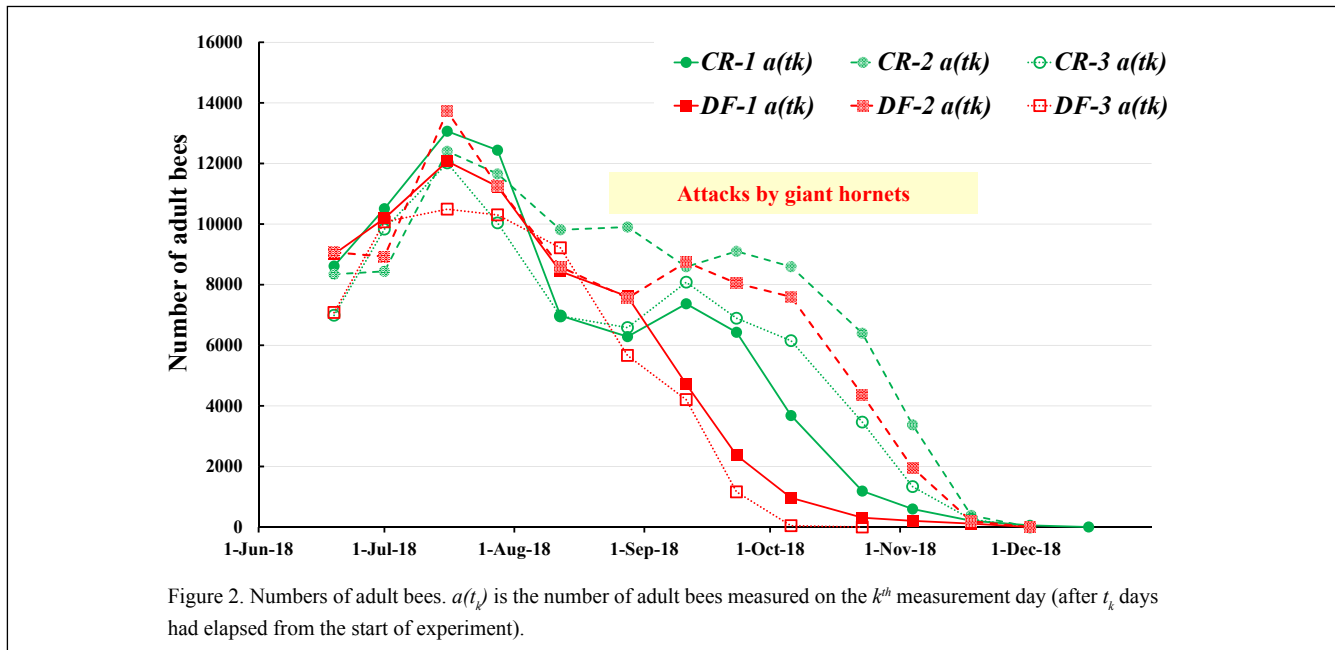


Table 1. Numbers of adult bees and capped brood. k is the measurement number, Δt_k is an interval between two successive measurement days [day], and t_k is the elapsed days from the start of experiment (June 19, 2018). $a(t_k)$ and $b(t_k)$ are the numbers of adult bees and capped brood that were measured on a measurement day, respectively. CR-1, CR-2, and CR-3 are the control colonies. DF-1, DF-2, and DF-3 are the dinotefuran (DF) exposed colonies where dinotefuran was administered via pollen paste under the same experimental conditions.

The queen bees of the control colonies remained in all colonies until the extinction of the colonies, but the queen bee in the DF-exposed colony disappeared before the extinction of the bee colony (DF-1, DF-3) or died (DF-2). The difference in the presence or absence of a queen bee between the control colony and the DF exposed colony was likely due to the DF-ingestion of the DF-exposed colony via PP containing DF. That is, since queen bees and their brood mainly consume pollen (PP), which is a source of protein, and do not consume much honey (SS), which is a source of energy, the PP containing DF might be the cause of the queen bee's active ingestion. In a bee colony without a queen bee (DF-1, DF-3), the number of bees cannot increase like that in the control colony in autumn. Around the start of the experiment, all colonies contained active bees, and it was thought that all colonies might overwinter. However, all the colonies became extinct on December 16, before or shortly after overwintering.

As can be seen from Figure 2, after the start of the experiment in mid-June (June 19), the number of adult bees in all bee colonies except DF-3 increased and began to decline after peaking in mid-July. This decrease is a common phenomenon in summer when there are few flowers in Japan. After that, the number of adult bees decreased in the second half of August and began to increase in September (these changes in numbers are often seen in Japan). However, in the second half of September, the numbers declined sharply, and by the middle of December, all the colonies had become extinct. This possibly occurred because the hornet catcher that was installed on each hive-box was removed due to the typhoon that hit in early September. Immediately after the typhoon, there were attacks by Japanese giant hornets,

so all the colonies suffered considerable damage. After this, a hornet catcher was again installed on each hive-box, but the colonies continued to suffer damage from the Japanese giant hornets.

In early October, the presence of *Varroa* mites in the hive-box was visually confirmed, and in the middle of November, the damage due to the mites was confirmed (Supplementary Table S5). Later, when expanding and examining the image taken, it was found that all colonies had contained mite-damaged bees from the start of the experiment. In previous experiments, there was no damage caused by ticks, so there was certainly a lack of attention in this experiment. It is thought that the extinction of the control colony occurred because the bee colony that was attacked by the mites rapidly weakened further due to the attacks from the Japanese giant hornets. From the middle of September until the beginning of December when the colony became extinct, although there was a difference in the number of adult bees, CR-2, CR-3, and DF-2, showed similar changes in the number of adult bees. CR-1 survived until December 16, longer than the other bees, even though the number of adult bees starting from mid-September was small. DF-1 and DF-3 had a much smaller number of bees in late August than other bee colonies, and there was no peak in the adult bee number in the middle of September followed by a decrease like that seen in the other bee colonies. These results will be discussed later.

The Number of Capped Brood

The measured value of the number of capped brood described in Table 1 is illustrated in Figure 3. From this figure, it can be seen that the number of capped brood in DF-3 decreased from July 1 to 4. This is probably because a new queen bee, with a sister relationship to the original, was placed in DF-3 on July 1 when the original queen bee could not be found, and bee eggs could not be laid during the periods when the queen bee was absent, as well as several days after new queen bee's placement. The numbers of capped brood in other colonies peaked at the beginning of July, earlier than the numbers of adult bees peaking mid-July. This seems to reflect a time lag due to the life cycle of the honeybee. Bee spawning was less significant in August due to the small number of flowers that produce honey. This is a

general trend. In September, the heat recedes, and flowers begin to bloom, so it is thought that the number of eggs laid increases. The rapid decrease in the number of eggs laid (the number of capped brood) in October is

different from the usual situation where the number of eggs laid begins to decrease around November. This is thought to be due to the attack of the Japanese giant hornets and the spread of mites, which is described later.

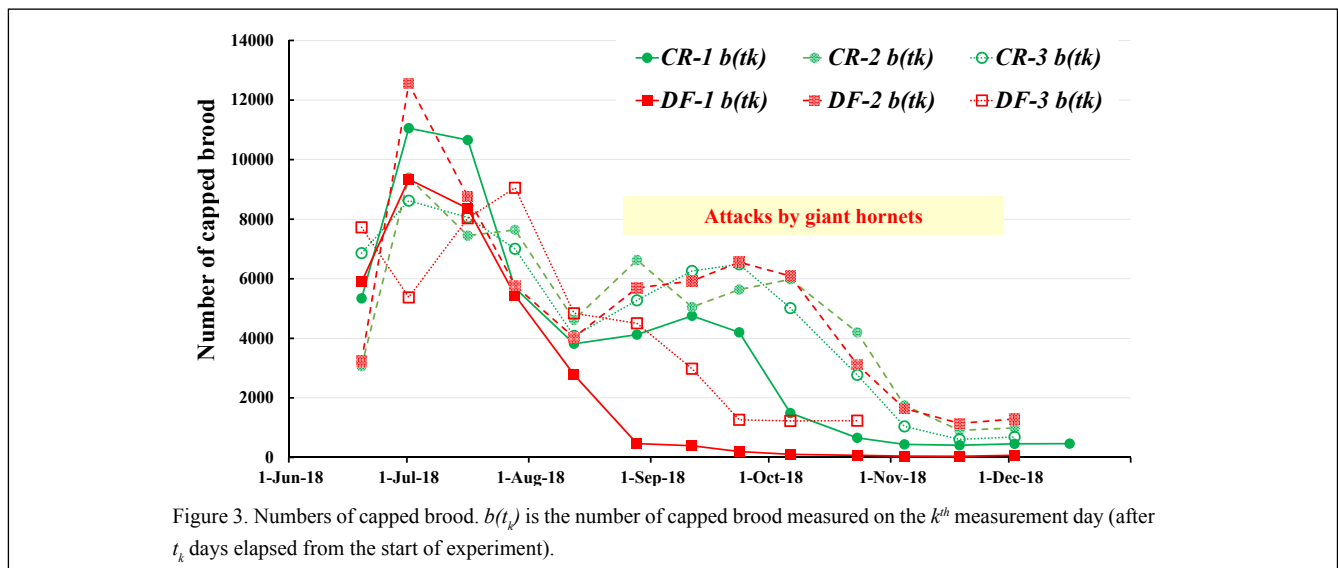


Figure 3. Numbers of capped brood. $b(t_k)$ is the number of capped brood measured on the k^{th} measurement day (after t_k days elapsed from the start of experiment).

Here, the individual bee colonies are examined in more detail. DF-1's increase in the number of capped brood at the start of the experiment (June 19) was at the same level as that of the other bee colonies, but there was no increase in the number of capped brood from mid-August to early October. This may be because the queen bee suffered from pesticides (DF) and mites, thereby reducing the number of eggs laid and also because the growth of the larvae was poor (the absence of the queen bee was confirmed on October 6). The number of capped brood (eggs laid) decreased dramatically from the beginning of September because the oviposition activity decreased sharply nearly a month before the queen bee disappeared. It should be noted that the eggs become capped brood about nine days after laying. CR-2, DF-2, and CR-3 also showed substantially similar changes in the number of their capped brood, peaking from mid-September to early October, after which the decreased bee colony became extinct (December 2). This tendency is very similar to the changes in the number of adult bees. The number of capped brood increased from mid-June to mid-July in DF-3, but the increase was less than that of the other bee colonies. However, the number of capped brood in DF-3 was similar to that of the other bee colonies and peaked in mid-July. However, a peak in the number of capped brood at the beginning of autumn, which is a normal decrease in a general bee colony, was not observed, and the number of capped brood from the beginning of autumn to winter decreased in the same way as that in DF-1. This trend is very similar to that in DF-1, which lost its queen bee on the same day in early October (October 6) but became extinct earlier than DF-1. It can be inferred that the earlier extinction of DF-3 compared to DF-1 occurred because the number of laid eggs (equivalent to the number of capped brood) after September was less than that in DF-1. CR-1, on the other hand, survived longer after mid-September, even though the number of its capped brood was smaller than that of CR-2, DF-2, and CR-3, as well as its number of adult bees. The cause of this phenomenon will be discussed later.

The Number of Dead Bees

In the four field experiments we conducted so far in this study (see Supplementary Table S1), dead bees were rarely observed even in the neonicotinoid-exposed colony, which was almost the same as the control

colony. In addition, the queen bees also remained until just before the extinction of the bee colony, exhibiting a similar trend related to CCD. After that, the neonicotinoid-exposed colony became extinct. From the field experiment results, it was estimated that neonicotinoids such as DF and clothianidin were the main causes of CCD. Moreover, the number of dead bees in the neonicotinoid-exposed colony, as long as the colony not attacked by Japanese giant hornets, was about the same as that of the control colony. The number of dead bees was 50 at most within the measurement period (7 days or 14 days). However, if a colony was attacked by Japanese giant hornets, more than 1,000 dead bees were sometimes observed. Since the influence of this factor on the experiment is large, during the queen bee activity period of the Japanese giant hornets in spring, many hornet traps were set around the experimental site with the cooperation of local people to capture the queen bees. At the time of the attacks of the Japanese giant hornets from the end of August, a hornet catcher was installed on the hive-box to reduce the damage caused by the hornets. In this experiment (2018 experiment), the same measures were again used against Japanese giant hornets. In mid-August, a hornet catcher was attached to each hive-box, but the hornet catcher was blown off by a typhoon at the beginning of September. Immediately afterward, major damage to the bee colony was caused by the giant hornets

Supplementary Table S3 shows the number of dead bees and the number of dying and dead Japanese giant hornets in each bee colony, where the number of dead bees includes the numbers of dead bees in the large tray installed under the hive-box and in the hive-box, and the number of dying and dead Japanese giant hornets includes the numbers of giant hornets in the large tray, in the hornet catcher, and in the hive-box. Each number in Supplementary Table S3 is the total number between two consecutive measurement days. These values are plotted in Figure 4. As can be seen from Figure 4, from the start of the experiment until the end of August, without a distinction between the control colony and the pesticide (neonicotinoid)-exposed colony, the number of dead bees totaled less than 20. However, the number of dead bees increased rapidly in September. This sharp increase may be due to the attacks by Japanese giant hornets that began in early September and continued until early December. These attacks caused a great deal of damage

to the bee colonies and seem to have affected the survival of the bee colonies. It should be noted that these Japanese giant hornet numbers represent

only a small part of the attacks, as many Japanese giant hornets returned to their nests without being captured by the catcher or killed by the bees.

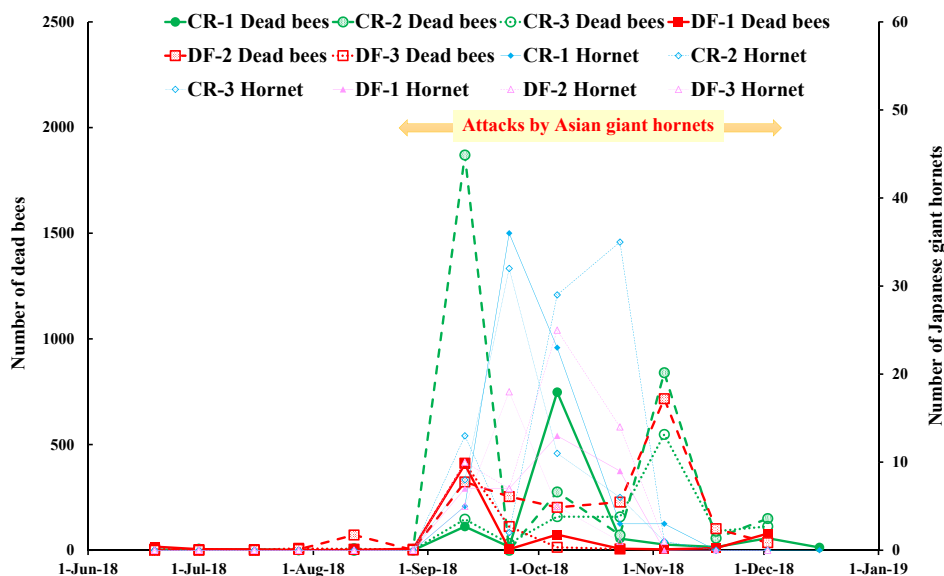


Figure 4. Number of dead bees. In this figure, the numbers of dead bees and Japanese giant hornets that attacked the bee colony between two consecutive measurement days are plotted.

Measurement Results of the Number of Adult Bees Damaged by *Varroa* Mites (Mite-Damaged Bees)

The presence of *Varroa* Mites was observed in early October, but it was only in mid-November that the bee colony was certainly being damaged by *Varroa* mites. Therefore, using the images taken, I investigated the damage to all the bees colony caused by the mites from the start of the experiment. As the most common method of quantifying damage due to *Varroa* mites, the number of mites in the bee colony was used. However, since it was impossible to count the total number of mites in each bee colony by enlarging the image (mites also were present in capped brood cells), each image was instead enlarged to count the number of bees with mites attached to them and the number of

bees with traces of mites (see Figure 1 for examples of adult bees damaged by mites). Table 2 shows the number of adult bees damaged by *Varroa* mites (mite-damaged bees) and the total number of adult bees (including the number of mite-damaged bees); the prevalence of adult bees damaged due to mites (mite-prevalence) is expressed by the ratio (percentage) of the number of mite-damaged bees to the total number of bees at each measurement day. The mite-damaged bees and the mite-prevalence are shown in Figure 5. In this experiment, since the number of adult bees was zero at the time of the bee colony extinction and it was not possible to determine the mite-presence, the mite-presence at the time of the colony's extinction is assumed to be one, based on the mite-presence just before the colony's extinction (See Figure 5).

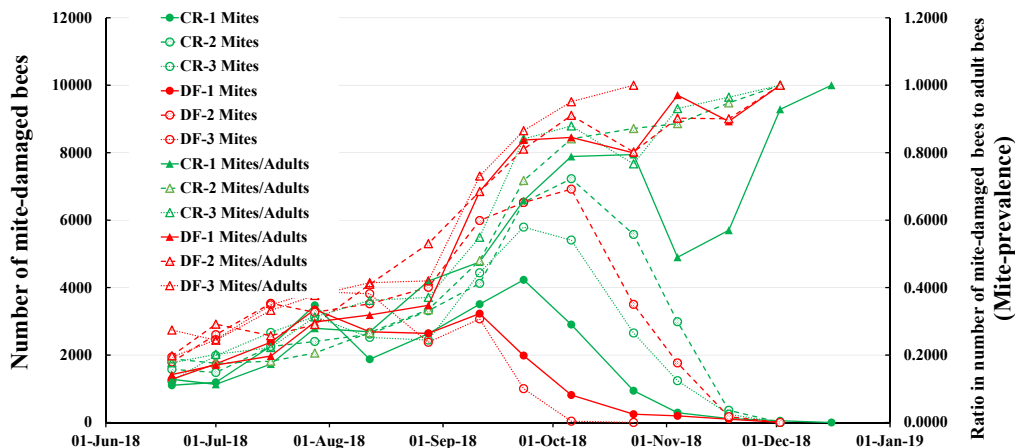


Figure 5. Number of mite-damaged bees and the mite-prevalence. The left vertical axis of the figure shows the number of mite-damaged bees on a measurement day, and the right vertical axis shows the mite-prevalence as the ratio of the number of mite-damaged bees to the total number of adult bees (mite-prevalence) on a measurement day. "CR-1 Mite" and "CR-1 Mites/Adults" denote the number of mite-damaged bees and the mite-prevalence in the CR-1 colony, respectively. The other keys also denote the numbers and mite-prevalences in each colony.

Table 2 Numbers of damaged bees by *Varoa* mites & adult bees and the ratio of number of the mite-damaged bees to number of adult bees at a measurement day in the 2018 experiment

Date of observation	Δt_k [day]	Elapsed day [day]		CR-1			CR-2			CR-3			DF-1			DF-2			DF-3		
		from exp. start	from DF start	Mites	Adults	M/A	Mites	Adults	M/A	Mites	Adults	M/A	Mites	Adults	M/A	Mites	Adults	M/A	Mites	Adults	M/A
19-Jun-18	0	0	-12	1103	8613	0.1281	1577	8343	0.1890	1237	6987	0.1770	1281	9010	0.1422	1795	9067	0.197970663	1940	7079	0.2741
01-Jul-18	12	12	0	1190	10507	0.1133	1482	8439	0.1756	1986	9830	0.2020	1739	10188	0.1707	2603	8926	0.291619987	2457	10071	0.2440
16-Jul-18	15	27	15	2263	13064	0.1732	2255	12402	0.1818	2670	12016	0.2222	2380	12070	0.1972	3532	13726	0.257321871	3488	10491	0.3325
28-Jul-18	12	39	27	3475	12439	0.2794	2401	11658	0.2060	3142	10037	0.3130	3351	11239	0.2982	3269	11252	0.290526129	3881	10301	0.3768
12-Aug-18	15	54	42	1876	6977	0.2689	2620	9812	0.2670	2523	6965	0.3622	2689	8437	0.3187	3519	8588	0.409757802	3819	9213	0.4145
28-Aug-18	16	70	58	2636	6284	0.4195	3313	9902	0.3346	2442	6583	0.3710	2643	7609	0.3474	4008	7558	0.530299021	2378	5662	0.4200
11-Sep-18	14	84	72	3507	7369	0.4759	4125	8589	0.4803	4438	8081	0.5492	3229	4716	0.6847	5988	8742	0.684969115	3072	4204	0.7307
23-Sep-18	12	96	84	4230	6427	0.6582	6531	9102	0.7175	5793	6891	0.8407	1985	2370	0.8376	6523	8046	0.810713398	1002	1158	0.8653
06-Oct-18	13	109	97	2903	3680	0.7889	7227	8593	0.8410	5408	6150	0.8793	815	964	0.8454	6919	7599	0.910514541	39	41	0.9512
23-Oct-18	17	126	114	943	1187	0.7944	5575	6395	0.8718	2654	3461	0.7668	247	309	0.7994	3502	4366	0.802107192	1	0	1.0000
04-Nov-18	12	138	126	292	596	0.4899	2989	3373	0.8862	1240	1332	0.9309	198	204	0.9706	1766	1957	0.902401635			
18-Nov-18	14	152	140	118	207	0.5700	363	383	0.9478	245	254	0.9646	100	112	0.8929	172	191	0.90052356			
02-Dec-18	14	166	154	52	56	0.9286	1	0	1.0000	1	0	1.0000	3	0	1.0000	1	0	1			
16-Dec-18	14	180	168	0	0	1.0000															

(1) The number of mite-damaged bees began to increase from the end of August but began to decline from the end of September to the beginning of October. This period of decreases in mite-damaged bees coincides with the time of the decrease in the number of bees and the number of capped brood. The mite-prevalence rapidly increased at the end of August and continued to increase even when the mite-damaged bees were reduced. The mite-prevalence approached 100% before colony extinction. The increases in the number of mite-damaged bees and the mite-prevalence at the end of August coincide with the start of the attacks by Japanese giant hornets. These increases seem to be due to the weakening of the bee colony caused by the attacks from Japanese giant hornets. The mite-prevalence continued to increase even when the number of mite-damaged bees decreased because the rate of the decrease to the total number of adult bees was higher than the rate of the decrease to the number of mite-damaged bees.

(2) Roughly speaking, the mite-prevalence was substantially the same regardless of the bee colony. However, CR-1 experienced a sharp decrease in mite-prevalence in early November for unknown reasons. It can be estimated that CR-1 survived longer than the other bees due to this. (3) The number of mite-damaged bees in the bee colony did not change starting from the beginning of October, with CR-2 > DF-2 > CR-3 > CR-1 > DF-1 > DF-3, which is the same as the order of the number of capped brood during this time. This can be understood as an increase in mites due to parasites on the larvae in the bee colony.

The Inside and Outside Temperatures of the Hive-Box

To determine the environmental conditions of the hive-box, the temperature and humidity data logger “EasyLog/EI-USB-2”, developed by Lascar Electronics Inc. (see <https://www.lascarelectronics.com/>), was used to measure and record the temperature inside and outside the hive-box every hour. It would have been better to measure the temperatures of all hive-boxes, but due to the costs, the data logger was placed inside CR-1, CR-3, DF-1, and DF-3 (farthest from the entrance at the bottom of the hive-box) and under the DF-2 hive-box (in the tray) to measure the outside temperature near the hive-box. Based on the data obtained from the data logger, CR-1 (TCR-1), DF-1 (TDF-1), and outside ambient temperature (T_a) were plotted

as shown in Figure 6. It can be seen from Figure 6 that the variation width of the temperature (TCR-1, TDF-1) in the hive-box was less than the variation width of the ambient temperature (T_a). The temperature in the hive-box was adjusted with about 30°C ambient temperature as the boundary. If the ambient temperature (T_a) was lower than 30°C, the temperature in the hive-box was higher than the ambient temperature (T_a), and if the ambient temperature (T_a) was higher than 30°C, the temperature in the hive-box was lower than the ambient temperature (T_a). Further, the temperature variation width of the DF-exposed colony (DF-1) was greater than that of the control colony (CR-1).

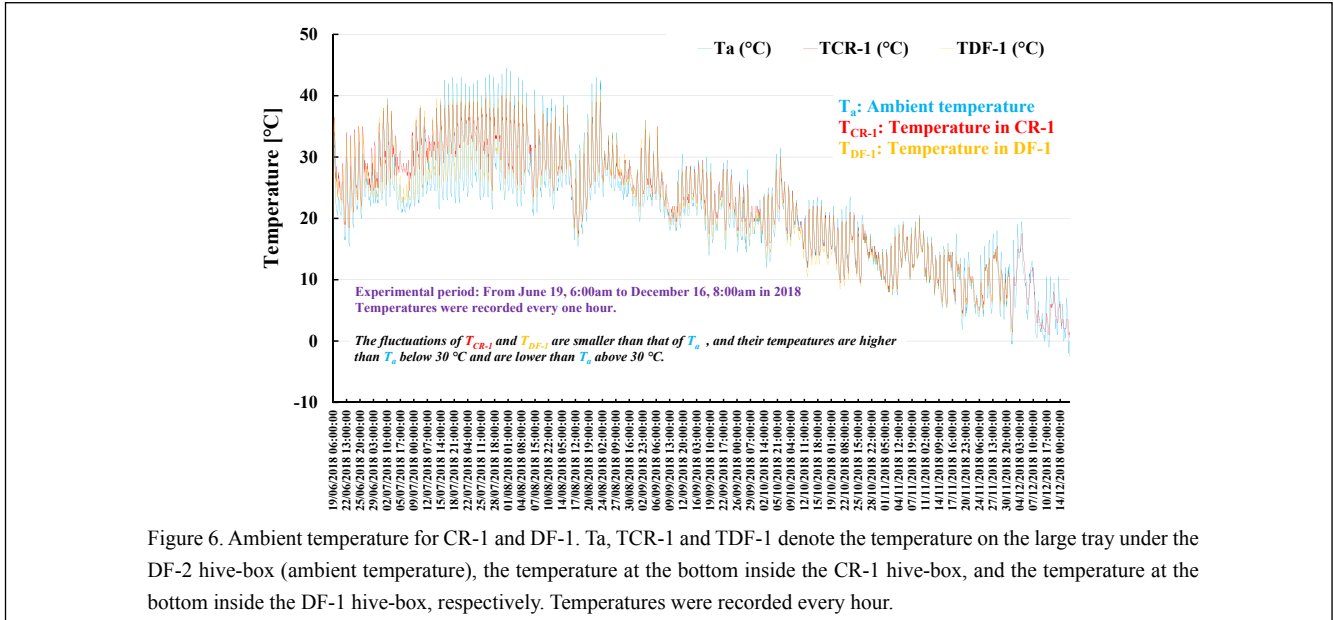


Figure 6. Ambient temperature for CR-1 and DF-1. T_a , TCR-1 and TDF-1 denote the temperature on the large tray under the DF-2 hive-box (ambient temperature), the temperature at the bottom inside the CR-1 hive-box, and the temperature at the bottom inside the DF-1 hive-box, respectively. Temperatures were recorded every hour.

Intakes of SS, PP, and DF

Next, the intakes (consumptions) of SS (SS_k), PP (PP_k), and DF (DF_k) in the honeybee colonies are considered between the $(k-1)^{th}$ and the k^{th} measurement days, where the start of experiment is $k=0$. SS_k and PP_k were obtained from each remaining amount measured on the k^{th} measurement day. DF_k can be calculated from PP_k with DF by considering the concentration of DF in PP (in this work, the concentration is 0.4 ppm). When the number of days between the $(k-1)^{th}$ measurement day and the k^{th} is Δt_k , the total number of honeybees in that period is $TNHB_k$. The average intake of SS per bee for Δt_k days ($AISSB_k$), the average intake of SS per bee for Δt_k days ($AIPPB_k$), and the average intake of DF per bee for Δt_k days ($AIDFB_k$) can be obtained from $SS_k/TNHB_k$, $PP_k/TNHB_k$, and $DF_k/TNHB_k$, respectively. Similarly, the average intake of SS per bee per day ($AISSBD_k$), the average intake of SS per bee per day ($AIPBBD_k$), and the average intake of DF per bee per day ($AIDFBD_k$) can be given by $SS_k/TNHB_k/\Delta t_k$, $PP_k/TNHB_k/\Delta t_k$, and $DF_k/TNHB_k/\Delta t_k$, respectively. Here, $TNHB_k$ can be given by the sum of the number of adult bees measured on the k^{th} measurement day and the number of new adult bees enclosed in pupae for Δt_k days. The adult bees measured on the k^{th} measurement day (initial adult bees) may have already ingested pesticides before the k^{th} measurement day. Therefore, two cases are considered in this paper: neglecting the pre-intake of the adult bees and considering the pre-intake of the adult bees.

Intakes of SS, PP, and DF when Neglecting the Pre-intake of Initial Adult Bees

Intake of SS when neglecting the pre-intake of adult bees Pesticide-free SS (800 g) was fed to every bee colony on the measurement day, and the consumption of SS was calculated from the remaining amount. The intake (consumption) of SS in each bee colony is shown in Table 3, which outlines the intake of SS per colony (SS_k) consumed over two consecutive measurement days (Δt_k) [g], the average intake of SS per bee ($AISSB_k$) for Δt_k days [mg/bee], and the average intake of SS per bee per day ($AISSBD_k$) for Δt_k days [mg/bee/day]. The following totals are provided in the last line of Table 3. Figure 7 shows the total number of adult bees in each bee colony and the average intake (consumption) of SS per bee, the average intake of SS per bee per day, and the intake of SS per bee per day in each measurement period [mg/bee/day] from the start of DF administration (July 1) until the extinction of the bee colony.

Table 3. Consumption of sugar syrup between two consecutive measurement days in each colony without consideration of sugar syrup already ingested by the initial honeybees.

Date	k	Δt_k [day]	CR-1				CR-2				CR-3				DF-1				DF-2				DF-3			
			TN HB _k	SS _k [g]	AIS SB _k	AISS BD _k	TN B _k	SS _k [g]	AIS SB _k	AIS SBD _k	TN B _k	SS _k [g]	AIS SB _k	AISS BD _k	TN HB _k	SS _k [g]	AIS SB _k	AISS BD _k	TN HB _k	SS _k [g]	AIS SB _k	AISS BD _k	TN HB _k	SS _k [g]	AIS SB _k	AISS BD _k
19-Jun-18	0	0																								
1-Jul-18	1	12	13954.0	0	0.00	0.00	11408.0	0	0.00	0.00	13848.0	0	0.00	0.00	14901.0	0	0.00	0.00	12308.0	0	0.00	0.00	14805.0	0	0.00	0.00
16-Jul-18	2	15	24327.0	800	32.89	2.19	20190.0	800	39.62	2.64	20606.0	800	38.82	2.59	21864.0	800	36.59	2.44	24612.0	800	32.50	2.17	16788.5	800	47.65	3.18
28-Jul-18	3	12	23721.0	800	33.73	2.81	19849.0	800	40.30	3.36	20076.0	800	39.85	3.32	20430.0	800	39.16	3.26	22481.0	800	35.59	2.97	18523.0	800	43.19	3.60
12-Aug-18	4	15	19549.0	800	40.92	2.73	21208.0	800	37.72	2.51	18794.5	800	42.57	2.84	18042.7	800	44.34	2.96	18463.3	800	43.33	2.89	21616.0	800	37.01	2.47
28-Aug-18	5	16	12066.3	800	66.30	4.14	15970.7	800	50.09	3.13	12402.3	800	64.50	4.03	12139.7	800	65.90	4.12	13961.3	800	57.30	3.58	15666.3	800	51.07	3.19
11-Sep-18	6	14	11096.5	800	72.09	5.15	17635.5	800	45.36	3.24	16010.8	800	49.97	3.57	8152.7	800	98.13	7.01	14196.3	800	56.35	4.03	10016.7	684	68.29	4.88
23-Sep-18	7	12	12124.0	800	65.98	5.50	13639.0	800	58.66	4.89	14343.0	800	55.78	4.65	5111.0	800	156.53	13.04	14670.0	800	54.53	4.54	7182.0	148.4	20.66	1.72
6-Oct-18	8	13	10978.1	523	47.64	3.66	15207.7	800	52.60	4.05	13918.6	800	57.48	4.42	2586.7	800	309.27	23.79	15151.6	800	52.80	4.06	2529.5	303	119.79	9.21
23-Oct-18	9	17	6790.8	228.3	33.62	1.98	17067.5	800	46.87	2.76	13254.6	598.4	45.15	2.66	1112.8	800	718.91	42.29	16237.9	674	41.51	2.44	1780.7	465	261.13	15.36
4-Nov-18	10	12	1845.0	50.8	27.53	2.29	10595.0	452.1	42.67	3.56	6234.0	90	14.44	1.20	379.0	44.4	117.15	9.76	7479.0	323.6	43.27	3.61				
18-Nov-18	11	14	1109.3	11	9.92	0.71	5411.2	121	22.36	1.60	2548.8	49	19.22	1.37	254.2	26.5	104.25	7.45	3877.3	174	44.88	3.21				
2-Dec-18	12	14	686.5	51	74.29	5.31	1448.2	0	0.00	0.00	962.2	12	12.47	0.89	157.5	4	25.40	1.81	1529.2	11	7.19	0.51				
16-Dec-18	13	14	592.7	0	0.00	0.00																				

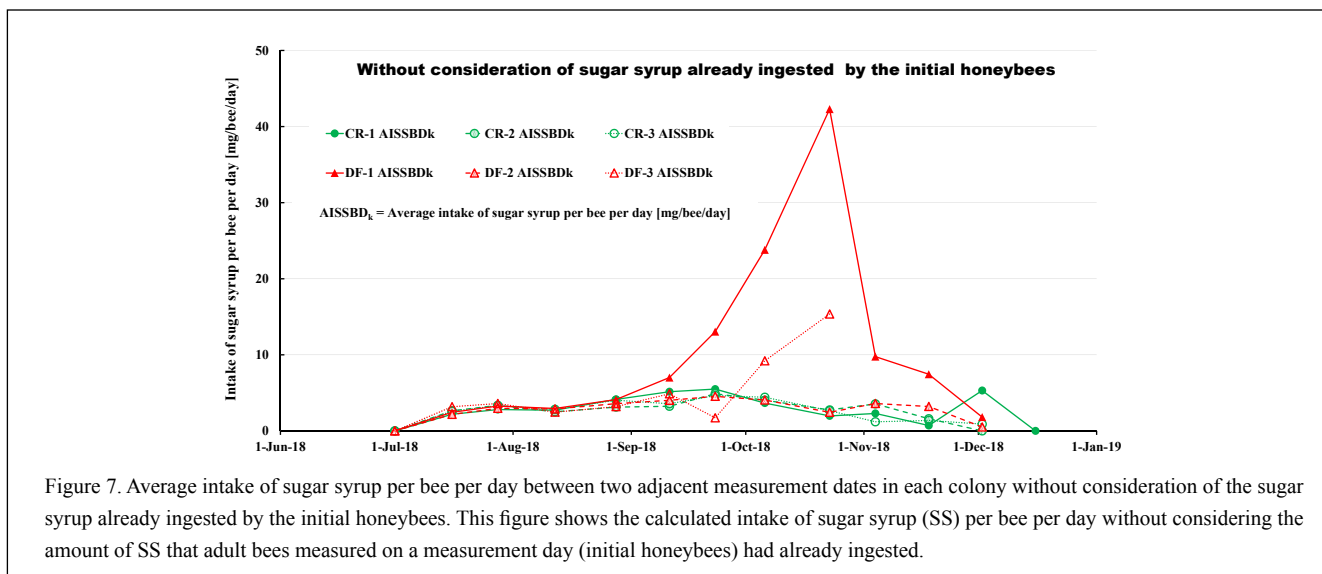


Figure 7. Average intake of sugar syrup per bee per day between two adjacent measurement dates in each colony without consideration of the sugar syrup already ingested by the initial honeybees. This figure shows the calculated intake of sugar syrup (SS) per bee per day without considering the amount of SS that adult bees measured on a measurement day (initial honeybees) had already ingested.

Table 3. Consumption of sugar syrup between two consecutive measurement days in each colony without consideration of sugar syrup already ingested by the initial honeybees. This table shows the calculated intake of sugar syrup (SS) between two consecutive measurement days without considering the amount of SS that the adult bees measured on a measurement day (initial honeybees) had already ingested. k = Measurement date number. Δt_k = Period between the two measurement date numbers of $k-1$ and k . CR-1, CR-2, and CR-3 = Control (pesticide-free) colonies. DF-1, DF-2, and DF-3 = Colonies (DF-exposed colonies) where dinotefuran was administered through pollen paste. $TNHB_k$ = Total number of honeybees involved in the consumption (intake) of sugar syrup during the period from the measurement date number of $k-1$ to that of k . $AISSB_k$ = Average intake of sugar syrup per bee between the measurement date numbers of $k-1$ and k [mg/bee]. $AISSBD_k$ = Average intake of sugar syrup per bee per day [mg/bee/day]. From June 19 (start of experiment) to July 1 (start of DF administration), 800 g of sugar syrup (pesticide-free) and 300 g of pollen paste (pesticide-free) were fed to every colony, but the consumption of sugar syrup and pollen paste was not recorded.

(1) The $AISSBD_k$ of the DF-exposed colony greater than the $AISSBD_k$ of the control (pesticide-free) colony. This result suggests that the excitable action of neonicotinoid pesticides such as DF may have compelled the bees to ingest more SS.

(2) The intake of SS per bee per day between Δt_k was about 4 mg/bee/day on average; in rare cases, it reached 10 times (DF-1).

(3) The intake of SS per bee from the start of DF administration (July 1) to the colony extinction (Total in Table 3) [mg/bee] was 80.33 for CR-1; 85.07 for CR-2; 77.35 for CR-3; 137.81 for DF-1; 79.51 for DF-2; and 80.14 for DF-3. With the exception of DF-1 (ca. 138 mg/bee), the DF-exposed colony, without distinguishing the control colony, was almost 80 mg/bee.

(4) The average intake of SS per bee per day (total) was 0.4782 mg/bee/day (CR-1); 0.5524 mg/bee/day (CR-2); 0.5023 mg/bee/day (CR-3); 0.8949 mg/bee/day (DF-1); 0.5163 mg/bee/day (DF-2); and 0.7030 mg/bee/day (DF-3). The average daily SS intake per bee (overall average in the experimental period) in the DF-exposed colony (0.7047 mg/bee/day) was higher than the average intake of the control colony (0.5110 mg/bee/day). Based on this fact, neonicotinoid pesticides (DF) may have an appetite-enhancing effect. DF possibly excites bees and boosts bee colony activity. Further, the average intake of DF per bee per day until the extinction of the DF-exposed colony showed a larger variation between the DF colonies than between the control colonies.

• Intake of PP when Neglecting the Pre-Intake of Initial Adult Bees

PP (300 g (mainly) or 500 g) was fed to each bee colony on each measurement day, and the intake (consumption) of PP was calculated from the remaining amount. The intake of PP of each bee colony ($AIPPB_k$) is shown in Table 4, which describes the same information given for SS. Similar to SS, the average intake of PP per bee per day in each measurement interval ($AIPPBD_k$) [mg / bee / day] is shown in Figure 8.

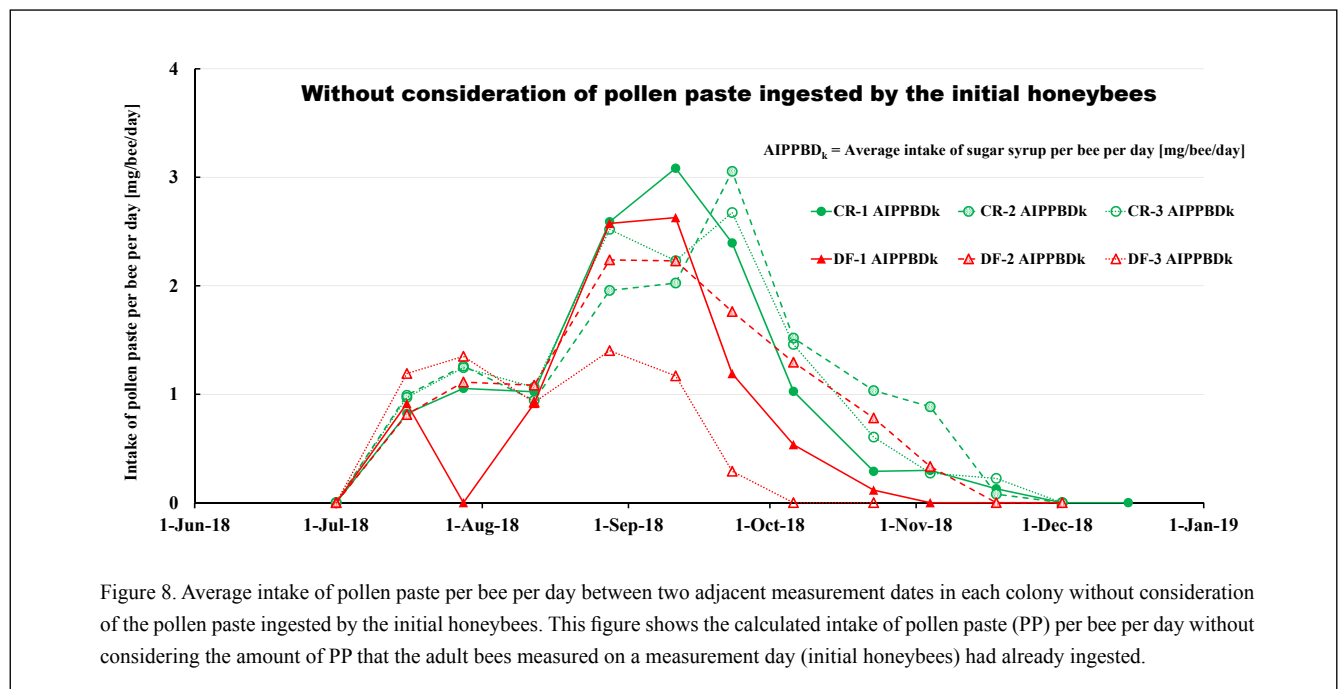


Table 4. Consumption of pollen paste between two consecutive measurement days in each colony without consideration of pollen paste already ingested by the initial honeybees.

Date	k	Δt_k [day]	CR-1				CR-2				CR-3				DF-1				DF-2				DF-3				Elapsed Days								
			TNHB _k	SS _k [g]	AIP PB _k	AIP- PBD _k	TN HB _k	SS _k [g]	AIP PB _k	AI PP BD _k	TN HB _k	SS _k [g]	AIP PB _k	AIP- PBD _k	TN HB _k	SS _k [g]	AIP PB _k	AI PP BD _k	TN HB _k	SS _k [g]	AIP PB _k	AI PP BD _k	TN HB _k	SS _k [g]	AIPPB _k	AIP PB D _k									
19-Jun-18	0	0																																	0
1-Jul-18	1	12	13954.0	0	0.00	0.000	11408.0	0	0.00	0.000	13848.0	0	0.00	0.000	14901.0	0	0.00	0.000	12308.0	0	0.00	0.000	14805.0	0	0.00	0.000	14805.0	0	0.00	0.000	12				
16-Jul-18	2	15	24327	300	12.33	0.822	20190.0	300	14.86	0.991	20606.0	300	14.56	0.971	21864.0	300	13.72	0.915	24612.0	300	12.19	0.813	16788.5	300	17.87	1.191	16788.5	300	17.87	1.191	27				
28-Jul-18	3	12	23721.0	300	12.65	1.054	19849.0	300	15.11	1.260	20076.0	300	14.94	1.245	20430.0	300	14.68	0.000	224810.0	300	13.34	1.112	18523.0	300	16.20	1.350	18523.0	300	16.20	1.350	39				
12-Aug-18	4	15	19549	300	15.35	1.023	21208	300	14.15	0.943	18794.5	300	15.96	1.064	18042.7	300	16.63	0.915	18463.3	300	16.25	1.083	21616	300	13.88	0.925	21616	300	13.88	0.925	54				
28-Aug-18	5	16	12066.3	500	41.44	2.590	15970.7	500	31.31	1.957	12402.3	500	40.32	2.520	12139.7	500	41.19	2.574	13961.3	500	35.81	2.238	15666.3	500	22.42	1.401	15666.3	500	22.42	1.401	70				
11-Sep-18	6	14	11096.5	479	43.17	3.083	17635.5	500	28.35	2.025	16010.8	500	31.23	2.231	8152.7	300	36.80	2.628	14196.3	443	31.21	2.229	10016.7	164	16.37	1.169	10016.7	164	16.37	1.169	84				
23-Sep-18	7	12	12124	348.4	28.74	2.395	13639	500	36.66	3.055	14343	460.4	32.10	2.675	5111	73	14.28	1.190	14670	310.2	21.15	1.762	7182	25	3.48	0.290	7182	25	3.48	0.290	96				
6-Oct-18	8	13	10978.1	146.5	13.34	1.027	15207.7	300	19.73	1.517	13918.6	264	18.97	1.459	2586.7	18	6.96	0.535	15151.6	255	16.83	1.295	2529.5	0	0.00	0.000	2529.5	0	0.00	0.000	109				
23-Oct-18	9	17	6790.8	33.4	4.92	0.289	17067.5	300	17.58	1.034	13254.6	136.3	10.28	0.605	1112.8	2.2	1.98	0.116	16237.9	215.7	13.28	0.781	1780.7	0	0.00	0.000	1780.7	0	0.00	0.000	126				
4-Nov-18	10	12	1845	6.6	3.58	0.298	10595	112.5	10.62	0.885	6234	20.4	3.27	0.273	379	0	0.00	0.000	7479	30	4.01	0.334									138				
18-Nov-18	11	14	1109.3	2	1.80	0.129	5411.2	6	1.11	0.079	2548.8	8	3.14	0.224	254.2	0	0.00	0.000	3877.3	0	0.00	0.000									152				
2-Dec-18	12	14	686.5	0	0.00	0.000	1448.2	0	0.00	0.000	962.2	0	0.00	0.000	157.5	0	0.00	0.000	1529.2	0	0.00	0.000									166				
16-Dec-18	13	14	592.7	0	0.00	0.000																									180				
Grand Total			70510.2	2415.9	34.26	0.204	81971.4	3118.5	38.04	0.247	82090.1	2789.1	33.98	0.221	46985.5	1793.2	38.16	0.248	85307.2	2653.9	31.11	0.202	59900.7	1440.3	24.04	0.211									

Bold-italic figures mean that the colony has been extinct on that day.

k = Measurement date number. Δt_k = Period between two measurement date numbers of $k-1$ and k . CR-1, CR-2, CR-3 = Control (pesticide-free) colonies. DF-1, DF-2, DF-3 = Colonies where dinotefuran is administered through pollen paste.

$TNHB_k$ = Total number of honeybees involved in the consumption (intake) of sugar syrup during the period from the measurement date number of $k-1$ to that of k .

$AIPPB_k$ = Average intake of pollen paste per bee between the measurement date numbers of $k-1$ and k [mg/bee]. $AIPPBD_k$ = Average intake of pollen paste per bee per day [mg/bee/day].

Table 4. Consumption of pollen paste between two consecutive measurement days in each colony without consideration of the pollen paste already ingested by the initial honeybees. This table shows the calculated intake of pollen paste (PP) between two consecutive measurement days without considering the amount of PP that the adult bees measured on a measurement day (initial honeybees) had already ingested. $AIPPB_k$ = Average intake of pollen paste per bee between the measurement date numbers of $k-1$ and k [mg/bee]. $AIPBD_k$ = Average intake of pollen paste per bee per day [mg/bee/day]. The other terms are provided in the legends of Table 3.

- (1) The average PP intake per bee between Δt_k was about 3 mg/bee/day at most.
- (2) The $AIPPB_k$ of the DF-exposed colony was less than the $AIPPB_k$ of the control colony. Except for DF-3, which was the earliest to become extinct, the difference was small.
- (3) The intake of PP decreased via the same trend as the decreases in capped brood approaching winter. Moreover, the intake of SS was different from the change in the intake of PP and not very related to the number of capped brood. This value was largely constant, and there was a tendency to decrease just before the colony's extinction.
- (4) The amount of PP consumed by one bee from the start of DF administration (July 1) to colony extinction was as follows (see "Total" at the last line of Table 4): 34.26 mg/bee (CR-1); 38.04 mg/bee (CR-2); 33.98 mg/bee (CR-3); 38.16 mg/bee (DF-1); 31.11 mg/bee (DF-2); and 24.04 mg/bee (DF-3). Roughly speaking, for the DF-exposed colony, without distinction from the control colony, the average intake per bee from July 1 to the colony extinction was 25–35 mg/bee. This fact shows that the bees did not avoid neonicotinoid pesticides such as DF and ingested the DF PP as much as pesticide-free PP. This result is consistent with our previous results [4].
- (5) The average amount of PP consumed in a day by one bee between July 1 and colony extinction was as follows: 0.204 mg/bee/day (CR-1); 0.247 mg/bee/day (CR-2); 0.221 mg/bee/day (CR-3); 0.248 mg/bee/day (DF-1); 0.202

mg/bee/day (DF-2); and 0.211 mg/bee/day (DF-3). Since there was little difference between the DF-exposed colony (ca. 0.220 mg/bee/day) and the control colony (ca. 0.224 mg/bee/day), neonicotinoid pesticides such as DF have no repellent effect but instead an appetite enhancement effect, which was also shown for SS.

• Intake of DF when Neglecting the Pre-intake of Initial Adult Bees

DF was administered to the DF-exposed colony from July 1 and continued to be administered until the bee colony became extinct. On the measurement day (about once / 2 weeks), new PP containing DF was administered, and the amount of DF ingested by the bees was calculated from the consumption of PP with pesticides and the concentration of DF in the PP. The control colony was also served the same amount of pesticide-free PP as the DF-exposed colony every time. In addition, 800 g of fresh pesticide-free SS was also fed to all the bee colonies each time. From the experimental data on two consecutive measurement days, the amount of DF ingested in each bee colony ($AIDFB_k$) was determined. From the results, the total amount of DF ingested by the bees, from the start of DF administration (July 1) to the end of the experiment, was calculated by dividing the total DF intake ingested per bee until the bee colony became extinct by the total number of adult bees involved in the DF intake (see the determination method in the previous paper [4]). These results are summarized in Table 5. The amount of DF ingested by the bees in two consecutive observation days ($AIDFB_k$) is plotted in Figure 9. Further, by dividing the average intake of DF per bee in the bee colony on the two consecutive measurement days ($AIDFB_k$) by the number of bees involved in DF intake at each measurement interval ($TNHB_k$), the average intake of DF that one bee would have consumed in one day in the measurement interval ($AIDFBD_k$) [ng/bee/day] was estimated. The intakes obtained in this way are shown in Table 6. Further, the average intake of DF per bee per day in the measurement interval ($AIDFBD_k$) shown in Table 6 is plotted in Figure 10.

Table 5. Intake of dinotefuran per colony from the start of experiment till colony extinction without consideration of pesticide brought-in by the initial honeybees

Measurement date	Intake of DF per colony between two consecutive days [$\mu\text{g}/\text{colony}$]					
	CR-1	CR-2	CR-3	DF-1	DF-2	DF-3
19-Jun-18	0.0	0.0	0.0	0.0	0.0	0.0
01-Jul-18	0.0	0.0	0.0	0.0	0.0	0.0
16-Jul-18	0.0	0.0	0.0	120.0	120.0	120.0
28-Jul-18	0.0	0.0	0.0	120.0	120.0	120.0
12-Aug-18	0.0	0.0	0.0	120.0	120.0	120.0
28-Aug-18	0.0	0.0	0.0	240.0	240.0	167.1
11-Sep-18	0.0	0.0	0.0	180.0	211.8	82.4
23-Sep-18	0.0	0.0	0.0	29.2	146.5	10.0
06-Oct-18	0.0	0.0	0.0	7.2	102.0	0.0
23-Oct-18	0.0	0.0	0.0	0.9	86.3	0.0
04-Nov-18	0.0	0.0	0.0	0.0	12.0	
18-Nov-18	0.0	0.0	0.0	0.0	0.0	
02-Dec-18	0.0	0.0	0.0	0.0	0.0	
16-Dec-18	0.0					
Total intake of DF per colony till colony extinction [$\mu\text{g}/\text{colony}$]	0.0	0.0	0.0	817.3	1158.6	619.5
Total number of honeybees till colony extinction	70510.2	81971.4	82090.1	46985.5	85307.2	59900.7
Intake of DF per bee till colony extinction [ng/bee]	0.00	0.00	0.00	17.39	13.58	10.34

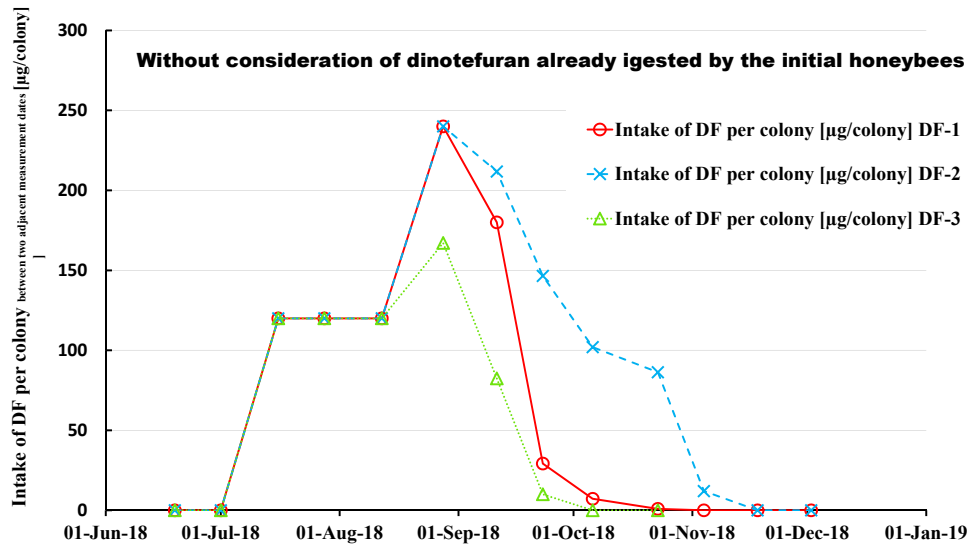


Figure 9. Intake of dinotefuran per colony without consideration of the dinotefuran already ingested by the initial honeybees. This figure shows the calculated intake of dinotefuran (DF) per colony without considering the amount of DF that the adult bees measured on a measurement day (initial honeybees) had already ingested.

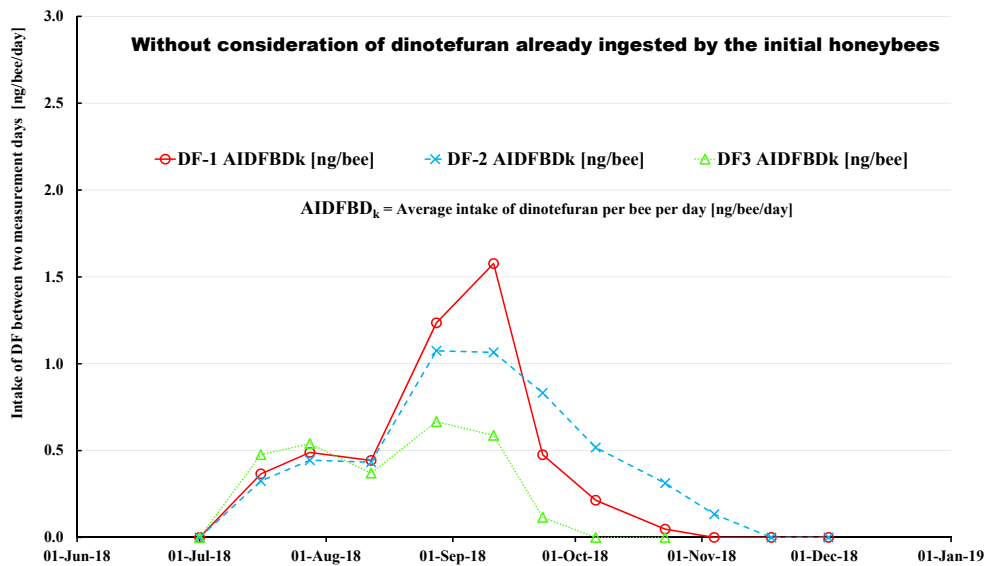


Figure 10. Average intake of dinotefuran per bee per day between two adjacent measurement dates (AIDFBD) without consideration of the dinotefuran already ingested by the initial honeybees. This figure shows the calculated intake of dinotefuran (DF) per bee per day without considering the amount of DF that the adult bees measured on a measurement day (initial honeybees) had already ingested.

Table 6. Intake of dinotefuran between two consecutive measurement days without consideration of dinotefuran already ingested by the initial honeybees.

Date	k	A_k [day]	DF-1				DF-2				DF3				
			DF _k [µg/bee]	TNHB _k	AIDFB _k [ng/bee]	AID-FBD _k [ng/bee/day]	DF _k [µg/bee]	TN- HB _k	AIDFB _k [ng/bee]	AID-FBD _k [ng/bee/day]	DF _k [µg/bee]	TNHB _k	AID-FB _k [ng/bee]	AID-FBD _k [ng/bee/day]	
19-Jun-18	0	0													0
01-Jul-18	1	12	0.0	14901	0.00	0.00	0.0	12308	0.00	0.00	0.0	14805	0.00	0.00	12
16-Jul-18	2	15	120.0	21864	5.49	0.37	120.0	24612	4.88	0.33	120.0	16789	7.15	0.48	15
28-Jul-18	3	12	120.0	20430	5.87	0.49	120.0	22481	5.34	0.44	120.0	18523	6.48	0.54	12
12-Aug-18	4	15	120.0	18043	6.65	0.44	120.0	18463	6.50	0.43	120.0	21616	5.55	0.37	15
28-Aug-18	5	16	240.0	12140	19.77	1.24	240.0	13961	17.19	1.07	167.1	15666	10.67	0.67	16
11-Sep-18	6	14	180.0	8153	22.08	1.58	211.8	14196	14.92	1.07	82.4	10017	8.23	0.59	14
23-Sep-18	7	12	29.2	5111	5.71	0.48	146.5	14670	9.99	0.83	10.0	7182	1.39	0.12	12
06-Oct-18	8	13	7.2	2587	2.78	0.21	102.0	15152	6.73	0.52	0.0	2530	0.00	0.00	13
23-Oct-18	9	17	0.9	1113	0.81	0.05	86.3	16238	5.31	0.31	0.0	1781	0.00	0.00	17
04-Nov-18	10	12	0.0	379	0.00	0.00	12.0	7479	1.60	0.13					12
18-Nov-18	11	14	0.0	254	0.00	0.00	0.0	3877	0.00	0.00					14
02-Dec-18	12	14	0.0	158	0.00	0.00	0.0	1529	0.00	0.00					14
16-Dec-18	13	14													14
Total intake of DF per colony till colony extinction [µg/colony]			817.3				1158.6				619.5				168
Total number of honeybees till colony extinction				46986				85307				59901			
Intake of DF per bee till colony extinction [ng/bee]					17.39				13.58				10.34		
Intake of DF per bee per day till colony extinction [ng/bee/day]						0.1035				0.0808				0.0615	

Table 5. Intake of dinotefuran per colony from the start of experiment until colony extinction without consideration of the pesticides brought in by the initial honeybees. This table shows the calculated intake of dinotefuran (DF) from the start of the experiment until colony extinction without considering the amount of DF that the adult bees measured on a measurement day (initial honeybees) had already ingested.

Table 6. Intake of dinotefuran between two consecutive measurement days without consideration of the dinotefuran already ingested by the initial honeybees. This table shows the calculated intake of dinotefuran (DF) between two consecutive measurement days without considering the amount of DF that the adult bees measured on a measurement day (initial honeybees) had already ingested. The terms can be seen in the legends of Table 3.

Note (1): Dinotefuran pesticide (DF) was administered through pollen paste as a vehicle starting from July 1, 2018.

Note (2): Pesticide-free sugar syrup (800 g) was newly fed into all colonies on every measurement date.

Note (3): The same amount of pollen paste without DF as that with DF fed into DF-exposed colonies was fed into all the control colonies.

Note (4): The same concentration and same amount of DF was administered into DF-1, DF-2, and DF-3. Note (5): 300 g of pollen paste with 0.4 ppm of DF was newly fed into every DF-exposed colony on each measurement date.

Note (6): 200 g of pollen paste with 0.6 ppm of DF was additionally fed into every DF-exposed colony on August 12, September 11, and September 23 due to concerns that there would be too little intake of DF per bee.

(1) The intake of DF ingested by each colony until bee colony extinction was 817.3 µg/colony (DF-1), 1158.6 µg/colony (DF-2), and 619.5 µg/colony (DF-3), and the intake of DF ingested by one bee was 17.79 ng/bee (DF-1), 13.58 ng/bee (DF-2), and 10.34 ng/bee (DF-3) (see Table 5). Taking the average value of the three colonies, the intake of DF per colony until was 865.1 µg/colony and 13.90 ng/bee when expressed as the intake per bee. These values are lower than the previous results of our field experiments.

(2) The intake of DF per colony increased rapidly starting in mid-August (Figure 9). This increase occurred because even though spawning began around autumn, the bee colony actively ingested PP containing DF due to the lack of blooming flowers. The amount of DF that one bee ingested per day in each measurement interval ($AIDFBD_k$) was, at most, 1.6 ng/bee/day and, in many cases, 0.5 ng/bee/day or less. In addition, the overall average intake of DF per bee per day from the start of DF-administration to colony extinction was less than 0.1 ng /bee/day.

The LD50 value of a bee is usually evaluated by pesticide intake within 72 hours (3 days) (contact, oral). In this study, the overall average daily intake of DF per bee for a 0.2 ppm administration concentration of DF was 0.1 ng/bee/day and 0.3 ng/bee/3 days for 3 days. This value is very low compared to the LD50 value of the DF value (7.6–75 ng/bee) for bees (see the previous paper [6]). Assuming that the measurement period of LD50 is 3 days and the relationship between the administration concentration of pesticides and the overall average pesticide intake per bee per day is correct, if the LD50 value is the lowest (7.6 ng/bee), the pesticide administration concentration is estimated to be about 5 ppm.

6.8.2. Intakes of SS, PP, and DF when Considering the Pre-intake of Adult Bees

The adult bees measured on the $(k-1)^{th}$ measurement day (initial adult bees) were considered to have already ingested SS, PP, and DF.

Assuming that the intake of SS, PP, and DF per bee on the $(k-1)^{th}$ measurement day is the average intake per bee from the $(k-2)^{th}$ measurement day to the $(k-1)^{th}$ measurement day ($AISSBD_{k-1}$, $AIPPPBD_{k-1}$, $AIDFBD_{k-1}$), each amount brought into the bee colony by the initial adult bees (pre-intake) to the k^{th} measurement day is determined by multiplying the average intake per bee between the $(k-2)^{th}$ and $(k-1)^{th}$ measurements by the number of initial adult bees on the k^{th} measurement day. The consumption (intake) per colony between the $(k-1)^{th}$ and k^{th} measurements can be obtained by the sum of the amount brought into the colony by the initial adult bees on the $(k-1)^{th}$ measurement day (pre-intake) and the consumption between the $(k-1)^{th}$ and k^{th} measurements calculated from the remaining amount measured on the k^{th} measurement day. Dividing each consumption (considering pre-intake) (SS_k , PP_k , DF_k) by the total number of honeybees ($TNHB_k$), we can obtain the average intake of the intake per bee and then divide it by the number of days between the $(k-1)^{th}$ and k^{th} measurement days (Δt_k), thereby obtaining the average intake of each bee per day.

Considering the pre-intake of the initial adult bees and ignoring the pre-intake, there was only a slight difference in each intake. Even if pre-intake was considered, the conclusions did not change when ignoring pre-intake. Therefore, in this paper, the details considering pre-intake are omitted.

Discussion

[Why did CR-1 Survive Longer than other Bee Colonies, Even though the Number of Adult Bees Since Mid-September \(Figure 2\) and the Number of Capped Brood \(Figure 3\) were Less than those in the Other Control Colonies? Further, Why did the Control Colony Fall out before the Winter?](#)

As can be seen from Figure 5, CR-1 is thought to have been the longest surviving colony because it had the lowest mite-prevalence. While most of the controls succeeded in overwintering in previous field experiments, in this experiment, even the control colony could not be overwintered because all bee colonies were attacked by mites before wintering (more than 80% mite-prevalence was observed as of October).

[Why did the Colony Exposed to DF via PP containing DF in this Experiment become Extinct despite having a Lower DF Intake than that Observed in Previous Field Experiments?](#)

Table 7. Outline of the pesticide intake in three field experiments in Shika, Japan. Location of the experimental site: mid-west Japan (Shika-machi) (Latitude 37°1'9"N; Longitude 136°46'14"N). CR-1, CR-2: Control colonies. CN: Clothianidin. DF: dinotefuran. FT: Fenitrothion. MT: Malathion. For example, "2011/2012 DF 1ppm/syrup" denotes the field experiment conducted from 2011 to 2012, where 1 ppm of dinotefuran was administered to bee colonies via sugar syrup. Bold text denotes the field experiments where dinotefuran was administered to bee colonies via pollen paste.

Table 7 summarizes the pesticide intake of the long-term field experiments. From Table 7, it can be seen that the intake of DF per bee whose colony became extinct by administering PP containing DF was 60–65 ng/bee (0.274–2.324 ng/bee/day) in previous field experiments (see the bee colonies highlighted in red in the table: 2011/2012 DF 0.565 ppm/pollen and 2011/2012 DF 5.65 ppm/pollen). In this study, however, the DF-exposed colony became extinct under a fairly small intake of 10–18 ng/bee (average: 14 ng/bee). This is estimated to have allowed the attacks of mites and Japanese giant hornets to collapse the bee colony despite a lower DF intake than before. Notably, since the colony exposed to DF via toxic PP in this study became extinct earlier than the control colony, it cannot be denied that the DF contained in PP also adversely affected the bee colonies. Further, since the mite-prevalence was higher in the DF-exposed colony than in the control colony, the DF contained in PP is considered to have accelerated the spread of mites. That is, the bee colony weakened by DF contained in PP experienced accelerated spread due to being attacked by mites. This supports the hypotheses that we previously published [1].

Table 7. Outline of pesticide intake in three field experiments in Shika, Japan1-3 Location of experimental site: mid-west Japan (Shika-machi) (Latitude 37°1'9"N; Longitude 136°46'14"N)

Colony name	Initial number of adult bees	Initial number of capped brood	Administration period of pesticide	Total intake of pesticide per colony during administration [mg/colony]	Total number of adult bees during administration	Total intake of pesticide per bee during administration [ng/bee]	Date of colony extinction	Data source
2011/2012 CR-1							Survived	[4]
2011/2012 DF 1ppm/syrup	2965	4263	9-Jul-11 to 21-Oct-11	4.208	13543	310.7	21-Oct-11	[4]
2011/2012 DF-0.565ppm/pollen	2158	2556	9-Jul-11 to 3-Dec-11	1.8692	30779	60.7	16-Feb-11	[4]
2011/2012 DF 10ppm/syrup	3296	3880	9-Jul-11 to 16-Jul-11	1.9	6344	290.3	17-Dec-11	[4]
2011/2012 DF 5.65 ppm/pollen	1659	6993	9-Jul-11 to 16-Jul-11	0.5257	3078	65.1	16-Jul-11	[4]
2012/2013 CR-1							Survived	[5]
2012/2013 CR-2							Survived	[5]
2012/2013 DF 2ppm/syrup	9173	9442	21-Jul-12 to 16-Aug-12	1.55	16524	93.8	16-Aug-12	[5]
2012/2013 FT 10ppm/syrup	8943	8732	21-Jul-12 to 16-Aug-12	17.97	19792	862.5	Survived	[5]
2013/2014 CR-1							7-Frb-14	[6]
2013/2014 CR-2							Survived	[6]
2013/2014 DF 0.2ppm/syrup	6017	2214	5-Sep-13 to 1-Dec-13	0.514	8612	56.7	5-Jan-14	[6]
2013/2014 CN 0.08ppm/syrup	7293	1506	5-Sep-13 to 1-Dec-13	0.4016	10130	39.6	5-Jan-14	[6]
2013/2014 FT 1ppm/syrup	10296	3232	5-Sep-13 to 28-Feb-14	3.86	19585	197.1	28-Feb-14	[6]
2013/2014 MT 1ppm/syrup	7676	1396	5-Sep-13 to 7-Feb-14	6.75	15724	429.3	7-Feb-14	[6]

Conclusions

A negative synergistic effect on bee colonies between DF and *Varroa* mites was determined based on the observation that colonies exposed to DF administered via pollen paste had smaller number of adult bees and became extinct earlier than the control (pesticide-free) colonies. Although the queen bees of DF-exposed colonies were lost in the middle of the experiment, those of the control colonies were present until the time of extinction. The rapid decrease in the number of capped brood in October was different from the usual situation in which the number of capped brood begin to decrease around November. This is likely due to the attacks of Japanese giant hornets and the spread of mites.

As the daily sugar-syrup (pesticide-free) intake of one bee in the colony exposed to DF administered via pollen paste was greater than that of the control colony, DF likely has an appetite-promoting effect. It is speculated that DF excites bees and increases the activity of ingestion.

The consumption of sugar syrup in one bee from the start of the DF administration to the extinction of the bee colony was almost 80 mg/bee, in both DF-exposed colonies and control colonies, except for DF-1, where the queen bee was lost early.

Since the amount of DF-containing pollen paste that one bee consumed in a day was almost no different between DF-exposed colonies where DF was administered via pollen paste and the control colonies, DF seems to have no repellent effect.

The amount of pollen paste consumed by one bee from the start of DF administration to the extinction of the bee colonies, regardless of the distinction between DF-exposed colonies and control colonies, was 25 to 35 mg/bee. The amount of DF per bee from the start of DF administration to the extinction of bee colonies was 10–18 ng/bee, and the daily intake of one bee was 0.1 ng/bee/day or less. Since the amount of DF per bee from the start of DF administration to the extinction of the bee colonies was 60–65 ng/bee in the 2011/2012 experiment [4] and 10–18 ng/bee in this work, the damage by mites seems to have greatly promoted colony extinction.

According to the seasonal changes in mite-prevalence, even when the number of mite-damaged bees began to decrease, mite-prevalence continued to rise and reached almost 100% at the time of bee colony extinction. This increase in mite-prevalence occurs because the rate of the decrease in the total number of adult bees is higher than the rate of the decrease in the number of mite-damaged bees. The number of mite-damage bees in the bee colony did not change since the beginning of October in the following order:

CR-2> DF-2> CR-3> CR-1> DF-1> DF-3. This order is the same as the order of the number of capped brood at this time. This was due to an increase in mites by parasites on larvae in the bee colony.

Since the mite-prevalence of honeybees increased rapidly from the beginning of September (Figure 5) and the apparent longevity of the adult bees began to increase around the end of September [46], it may be effective to exterminate *Varroa* mites for bee larvae in early autumn (early September) when the preparations for wintering have already been started.

The fluctuation range of the inner temperature of the hive-box (T_i) was smaller than that of the ambient temperature of the hive-box (T_a), while the fluctuation range of the inner temperature of the DF-exposed colony hive-box was larger than that of the control colony hive-box. The T_i was controlled with around 30°C of T_a as the boundary. If T_a was 30°C or less, T_i became higher than T_a , and if T_a was 30°C or more, T_i was lower than T_a .

In this study, it was found that the bee colonies collapsed via the intake of a smaller amount of DF due to the synergistic effect of DF (a neonicotinoid pesticide) and mite-damage. To prevent a bee colony collapse, beyond minimizing the adverse effects of neonicotinoids with long-term residual effects and high insecticide properties on the bee colony, it is necessary to reduce the damage from mites as much as possible.

In this paper I have reported the seasonal changes in the bee colony size and mite-prevalence of dinotefuran-exposed and mite-damaged honeybee colonies. This study found that not only neonicotinoids but also *Varroa* mites can adversely affect honeybee colonies.

In the near future, using a mathematical model that was previously proposed [46], I will investigate the seasonal changes in apparent longevity among DF-exposed and *Varroa*-mite-damaged colonies in which DF was administered via pollen paste using the numbers of adult bees and capped brood measured accurately in this work.

Supplementary Materials

Figure S1: The inside of the hive box numbered clockwise from the face of the entrance; Figure S2: An example of a numbered comb frame; Figure S3: Photo stand, two empty hive-boxes, experiment log and beekeeping tools; Figure S4: Overall view of the experimental site; Method S1: Field experimental procedures; Method S2: Determination procedure for the numbers of adult bees and capped brood; Method S3: Criteria for determining mite-damaged bees and estimating the number of mite-damaged bees; Table S1: Summary of the experimental conditions and results of four long-term field experiments in Shika, Japan; Table S2: Weather information and Experimental time; Table S3: Summary of observational results for the control colonies (CR-1; CR-2; CR-3); Table S4: Summary of the observational results for DF-exposed colonies (DF-1; DF-2; DF-3); Table S5: Summary of experimental notes.

Author Contributions

Toshiro Yamada conceived and designed the experiments, performed the experiments, analyzed the data, prepared the figures and/or tables, authored and reviewed drafts of the paper, and approved the final draft.

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Conflicts of Interest

The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results. Incidentally, the author has absolutely nothing to do with Yamada Bee Farm Inc. though “Yamada” is same name.

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Supplementary materials: Methods, Tables & Figures

Seasonal changes in size and mite-prevalence of a bee colony exposed to dinotefuran via pollen paste and damaged by *Varroa* mites

[Short title]

DF-exposed & mite-infested bee-colony

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List of Supplementary Materials

Supplementary Method S1. Field experimental procedures.

Supplementary Method S2. Determination procedure of the numbers of adult bees and capped brood.

Supplementary Method S3. Criteria for determining mite-damaged bees and estimating the number of mite-damaged bees.

Supplementary Table S1. Summary of experimental conditions and results of four long-term field experiments in Shika, Japan.

Supplementary Table S2. Weather information & Experimental time.

Supplementary Table S3. Summary of observational results for control colonies (CR-1; CR-2; CR-3).

Supplementary Table S4. Summary of observational results for DF-exposed colonies (DF-1; DF-2; DF-3).

Supplementary Table S5. Summary of experimental note.

Supplementary Figure S1. The inside of the hive box is numbered clockwise from the face of the entrance.

Supplementary Figure S2. An example of numbered comb frame.

Supplementary Figure S3. Photo stand, two empty hive-boxes, experiment log and beekeeping tools.

Supplementary Figure S4. Overall view of experimental site.

Supplementary Method S1. Field experimental procedures.

STEP 1) To avoid being stung by a bee, dress firmly with protective measures, and conduct experiments. Further, the PP to be used, so as to be able to see what is administered to any bee colony, CR-1 (black) and DF-1 (red) or the like in magic ink it is described on a tray containing PP.

STEP 2) Experimental tools, recording paper, SS, trays with PP, camera, to prepare the empty hive-box or the like.

Step 3) Take a picture of the experiment site (entire hive-box) before the experiment (check whether abnormal conditions have occurred).

STEP 4) Count the number of dead bees in a large tray placed under the hive-box while picking up with tweezers. Throw away what you've finished counting out of the tray. It should be noted that the number of dead bees in the hive-box is counted during the internal inspection of the hive-box.

STEP 5) Take a picture of the front of the hive-box before the internal inspection (record and status record to make sure that the experiment target of the photography is not wrong) ([Photo S1-1](#)).

STEP 6) Open the hive-box and take the cloth that covered the top of the comb frame of the hive-box, and take a whole photograph of the top of the hive-box ([Photo S1-2](#)). In addition, gently return the bee attached to the cloth in the hive-box.

STEP 7) The tray with PP and the container with SS (tray, feeding frame) are taken out from the hive-box, measuring the remaining amounts of PP and SS by the upper dish balance. Incidentally, when taking out the container from the hive-box, gently return the bee attached to the container in the hive-box.

STEP 8) Gently remove the comb frame with the bee from the hive-box in the order of number (in order from left to right toward the front of the hive-box), take a picture of both sides of each comb frame ([Photo S1-3](#)). When you are taking a picture, if you find a queen bee, after taking a picture of the queen bee ([Photo S1-4](#)) put it in the queen cage and once isolate it. When the photograph of the comb frame is finished, put the comb frame in the empty hive-box prepared beforehand, and the lid of the hive-box is closed. The queen bee in the queen cage is placed at the top of comb frame placed in an empty hive-box.

STEP 9) After taking pictures of both sides of all the hive-frames with bees, take pictures of the four sides and the bottom in the hive-box in order to determine the number of adult bees left in the hive-box ([Photo S1-5](#)). If you can't find a queen bee when you're taking a picture of a comb frame, carefully check for the presence or absence of the queen bee, and if so, take a picture. If adult bees are outside the hive-box as well as inside the hive-box (sometimes near the entrance of the hive-box at tropical nights), take a picture to count the number ([Photo S1-6](#)).

STEP 10) If you cannot find the queen bee by all means, check the queen bee on all the comb frames carefully before taking a picture of capped brood of the next operation.

STEP 11) Take out the comb frame with bees that had been placed in the spare empty hive-box in the number order, return all the bees attached to the comb frame to the original hive-box. After taking pictures of both sides of the comb frame without bee in the order of the comb frame number [Photo S1-7](#)), the comb-frame is also returned to the original position of the original hive-box.

STEP 12) When taking a picture of the comb frame without bee, you may discover some abnormality, such as queen-cells, *Varroa* mites, wax-moth larvae and so on. In this case, keep taking pictures of them. These anomalies are not limited to this situation, and if found, record them in photographs as evidence ([Photo S1-8](#)).

STEP 13) If the queen bee is not found by all means, check in the next experiment. If it is not found next time, by the presence or absence of queen cages and by observing in detail the situation of spawning, etc., the presence or absence of a queen bee is determined.

STEP 14) After covering the cloth on the top of comb frames, put new SS (800 g) and PP (300 g) in the corresponding hive-box, the measurement is ended with the lid of the hive-box.

STEP 15) After completion of the experiment, the test contents are confirmed and described in the research note.

Photo. S1-1. Front view of the hive-box (Example of CR-1).



Photo. S1-2. Top photo of the hive-box (Example of DF-1).

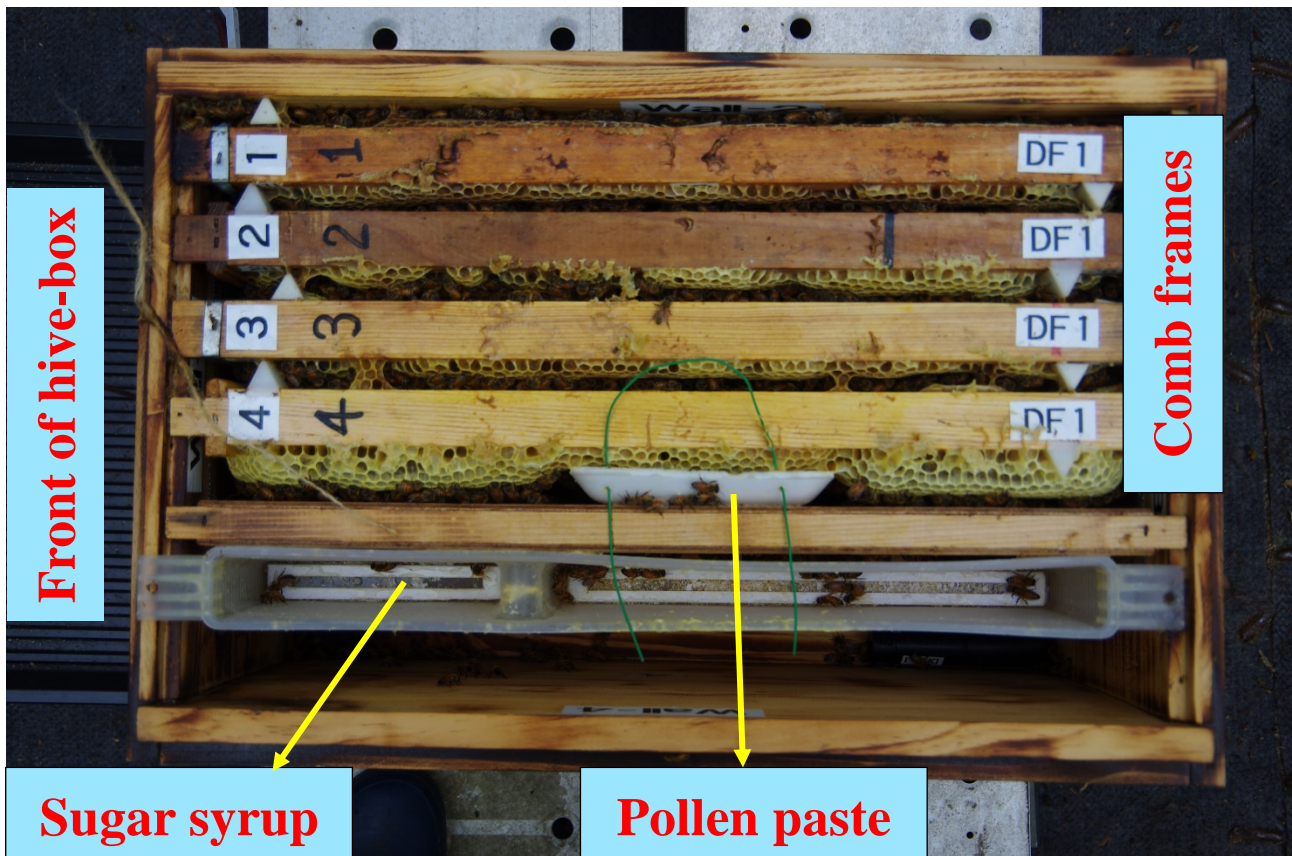


Photo. S1-3. Comb-frame photo with bees (Example of 3F in CR-1).



Photo. S1-4. Queen bee (Example in CR-1).



Photo. S1-5. The inside photos of the hive-box composing of four walls and bottom (Example of bottom).

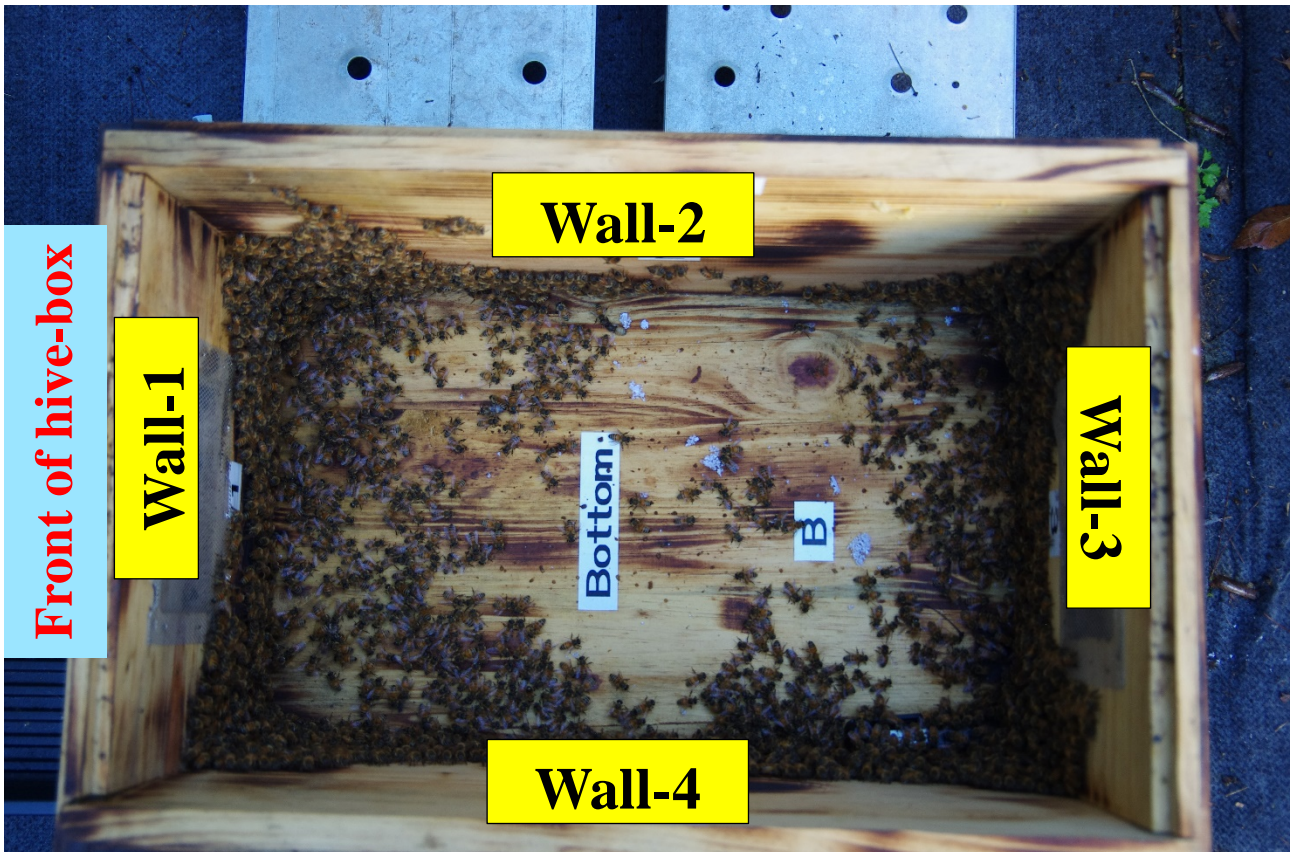


Photo. S1-6. Outside photo of the hive box with bees.



Photo.S1-7. Comb-frame photo without bees after removing bees from the comb (Example of 3F in CR-1).



Photo. S1-8. Unusual situation discovery (Example of queen-cell & wax-worm in DF-1).



Supplementary Method S2. Determination procedure of the numbers of adult bees and capped brood.

STEP 1) The image of comb is binarized, and then the difference in brightness between the adult bee or the capped brood in the comb image and the place where there is no bee, and the features of the adult bee and the capped brood are used to identify the bee and the capped brood. The identified adult bee or capped brood is counted.

STEP 2) In order to count as accurately as possible, before the binarization process of the image, divide the entire image into several areas that seem to have the same threshold value in each area (up to 4 areas) as below. Then, enter the optimal threshold for each divided area (the maximum number of divided areas 4, the split shape is optional). Examples of division: (1) Distinguish between the capped honey area and the capped brood area. (2) Distinguish between areas where there are few bees and areas where bees are dense. (3) Distinguish between different areas of contrast or lightness (e.g., the part where the light hits and the part of the shadow).

STEP 3) Enter the type of key to mark counted adult bees and capped brood one by one (+, *, numbers, etc.) and the color and size of key ((you can change them after the end of the count).

STEP 4) When the count is performed, while automatically marking those counted bee or capped brood in the image, continue counting, the number of counts at that time is displayed at the bottom of the image. After counting the adult bee or capped brood in the image in a short time, the total count in the image is displayed at the bottom of the image. The count status is visually determined, if dissatisfied, by changing the threshold value, recount. In a few tries, even if you change the threshold, if there is no improvement in the count situation, the count of approximate number by automatic measurement in the computer is assumed to have been completed.

STEP 5) After automatic counting by the computer, using the same software, switch to manual count operation, and then correct the count error at the time of automatic counting. Therefore, using a large screen monitor, while further enlarging the image, mark-mistakes of adult bees and capped brood during automatic counting (duplicate count, counting things that are not the object) and forgetting to mark (due to the overlap of bees, the blurred image, the extreme differences in light and dark, etc.) are corrected while visually checking, and all possible efforts are exerted to obtain the number of adult bees or the number of capped brood as accurate as possible (most important and serious work). It should be noted that, even when the software is manual counting, by the removal and grant of the mark, automatically change the number of bees or capped brood. You can resume this fix at any time. If it is determined that the correction is complete, it moves to the measurement of the next new image.

STEP 6) When posting the measured number of adult bees or capped brood in a separate table, call the measured image, check again in the enlarged image, after checking whether there is a count error (correct if you find a mistake), the number is posted to the separate table. The sum total of the numbers of adult bees on all combs and the numbers of adult bees on four walls and bottom in the hive-box, or the sum total of the numbers of capped brood of all combs, is the number of adult bees or the number of capped brood in a bee colony at a measurement day, respectively.

Supplementary Method S3. Criteria for determining mite-damaged bees and estimating the number of mite-damaged bees.

In this study, rather than a method of directly counting the number of *Varroa* mites in the bee colony, it is assumed that the number of bees damaged by mites is equivalent to the degree of the bee colony damage caused by mites. The number of mite-damaged bees on the enlarged photographic image of the comb with bees taken to measure the number of adult bees, using the software developed to count the numbers of adult bees and capped brood (see [Table S2](#)), is manually counted while referring to [Figure 4](#) according to the following criteria.

In this method, it is not possible to directly evaluate the absolute number of mites in the bee colony, but it is possible to estimate indirectly the damage to the bee colony caused by mites. Additionally, since a photographic image for the measurement of the number of adult bees in a field experiment is used to evaluate the damage to the bee colony caused by *Varroa* mites, it is possible to re-measure the number of mite-damaged bees while checking again and again whenever you need, and it is possible to improve the measurement accuracy. Further, using the photographic image for the past adult number measurement, it is also possible to measure the number of mite-damaged bees during past experiments.

Notes and criteria for determining whether the damaged bee by the *Varroa* mite using the captured image are shown below.

Female *Varroa* mites enter the comb-cell where the larva is just before capping the cell, lay the egg (all male mites) and the number of mites increases in the capped comb-cell. Not only the larvae, it is said to continue to cause damage to adult bees. However, it is impossible to count the number of mites in the capped comb-cell. Therefore, although indirect, there is a trace of mite-damage bees (the presence of mites can be confirmed, there are traces of swelling and inflammation that seem for the bee to have been bitten by mites, wings of the bee are deformed or fallen out, there is a missing mark of the mite on the bee), and the number of adult bees having the above traces can be regarded as an index to estimate the mite-damaged bee in the bee colony. The specific criteria for judgment are shown below.

It is suspected that the three-dimensional one attached to a bee, which is small and round (including the ellipse), is a mite in the color of the red-brown system (including the oval) and red-brown system (including the semi-transparent). A suspected mite are determined whether it is a mite based on the criteria below and [Figure 4](#), while being examined from various angles, using an enlarged image. If a suspected mite cannot be determined by all means, it is considered a mite or a trace of the mite.

- 1) What appears to be planar rather than three-dimensional in the vicinity of the neck is considered to be an innate pattern of a bee appearing when the bee bends its neck or a shadow of other bee wings, etc., and is not considered damage by mites.
- 2) Small colors other than red-brown and black (especially whitish ones) are regarded as pollen.
- 3) What the wings appear three-dimensional like wrinkles deformed is regarded as a shadow due to the shrinkage of the wings. However, the cause of the shrinkage of the wings is a large

possibility of deformed wing virus mediated by *Varroa* mites. The state of the shrinkage of the wing is scrutinized on the image to determine whether the shrinkage is due to mites.

- 4) If a bee with shrunk or missing wings has traces of a mite bite, it is considered a mite-damaged bee, even if it is not possible to confirm the presence of a mite.
- 5) If you can't distinguish between mites and pollen or garbage, you should consider them mites. However, if it is clear that it is other than mites such as pollen and garbage, exclude it.
- 6) Planar ones are usually determined to be not mites. However, after scrutiny, exceptionally, it may be considered a mite-damaged bee.
- 7) Except for those that seem to be the rise of muscles, etc.
- 8) The difference between a mite and garbage is judged by shape, color, surrounding situation, etc., and it counts unnatural things like mites (although it is subjective, counting the one like the mite)
- 9) Traces bitten by mites (swelling by the bites) are also thought to be damage to bees.
- 10) Regardless of what appears to be a mite or the number of traces bitten by mites, it is treated as a mite-damaged bee.
- 11) Traces of a mite fallen out from a bee are treated as a mite-damaged bees.

Just before the larva's cell is capped, the mites lay eggs in the food in the cell, since the eggs grow while sucking the body fluid of the larva hatched in the capped cell, it is almost impossible to find almost a mite even in the open cell. Therefore, it was decided not to evaluate the mites in the cells.

Mites are present in various places in the hive-box, shape is also easy to mistake for garbage and pollen, etc., also present in the invisible place (especially in the comb-cell), although the absolute number of mites is not known, this method to evaluate by the number of adult bees damaged by mites is believed to be a qualitative indicator of the number of presence of mites. Although the number of mites in the cell cannot be known, the infestation state of mites in the bee colony will be reflected in the number of mites adhering to the adult bee.

This method includes an uncertain element, under the above criteria, by measuring in the same person, and relative comparison of the number of mites between each bee colony, to some extent the time course of each bee group it is considered to represent. By the way, whenever a doubt occurs, again, using the image for the adult bee number measurement taken, recount.

Supplementary Table S1. Summary of experimental conditions and results of four long-term field experiments in Shika, Japan.

Experiment name (Experimental period)	2011/2012 experiment From July 9, 2011 to April 2, 2012 (268 days)	2012/2013 experiment From June 28, 2012 to July 26, 2013 (393 days)	2013/2014 experiment From Aug. 13, 2013 to Feb. 28, 2014 (199 days)	2018 experiment (This work) From Jul. 19, 2018 to Dec. 16, 2018 (180 days)
Object of Study	To investigate the difference in the long-term impact on a bee colony between toxic sugar syrup (honey) as an energy source and toxic pollen paste (bee bread) as a protein source which are exposed to a neonicotinoid	To investigate the difference in the long-term impact on a bee colony between a neonicotinoid and an organophosphate which are administered through sugar syrup	To investigate the difference in the long-term impact on a bee colony between a neonicotinoid and an organophosphate which are administered through sugar syrup under lower concentrations than in the previous work	To investigate the seasonal changes in colony size and mite-prevalence in a honeybee colony exposed to dinotefuran via pollen paste and infested with <i>Varroua</i> mites
Circumstances around Experimental Site				
Experimental site (latitude and longitude)	Shika (mid-west Japan) (37°19' N, 136°46'14" E, 43m above sea level)	Same as the left	Same as the left	Shika (mid-west Japan) (37°12'35" N, 136°45'38" E, 70m above sea level)
Limitation of honeybee activities	Honeybees can freely forage about for food in a hive or in fields	Same as the left	Same as the left	Same as the left
Environmental effects of pesticides other than administered pesticides	A pesticide-free watering place & a pesticide-free field of flowers in the apiary	Same as the left	Same as the left	Same as the left
Aerial-crop-dusting farmland near experimental site	Nothing	Nothing	Nothing	Nothing
Seasonal changes	Distinct	Distinct	Distinct	Distinct
Experimental Conditions				
Initial numbers of apiaries, colonies and combs (frames)	One apiary (private), five colonies per apiary, three combs per colony (one colony for each same conditions)	One apiary (private), four colonies per apiary, three combs per colony (one colony for each same conditions except 2 controls)	One apiary (private), six colonies per apiary, three combs per colony (one colony for each same conditions except 2 controls)	One apiary (private), six colonies per apiary, three combs per colony (3 control colonies & 3 dinotefuran-exposed colonies)
Initial number of bees per colony	1700 to 3400 bees (accurately counted from photos)	5600 to 7100 bees (accurately counted on photos)	5400 to 7600 bees (accurately counted on photos)	7000 to 9000 bees (accurately counted on photos)
Initial number of capped brood per colony	2600 to 6100 capped brood (accurately counted from photos)	4000 to 5700 capped brood (accurately counted from photos)	4200 to 7600 capped brood (accurately counted from photos)	3100 to 6900 capped brood (accurately counted from photos)
Kind of pesticide	Dinotefuran	Dinotefuran, Fenitrothion	Dinotefuran, Clothianidin, fenitrothion, Malathion	Dinotefuran
Concentration of pesticide	Dinotefuran: 1 & 10 ppm in sugar syrup, 0.565 & 5.65 in pollen paste	Dinotefuran: 2 ppm in sugar syrup, Fenitrothion: 10 ppm in sugar syrup	Dinotefuran: 0.2 ppm in sugar syrup, Clothianidin: 0.08 ppm in sugar syrup, Fenitrothion & Malathion: 1 ppm in sugar syrup	Dinotefuran: 0.4 ppm in pollen paste
Origin of a queen	Unknown in detail (<i>Apis mellifera</i>) Bee colonies purchased from a bee farm	Same as the left	Same as the left	Queen bees in sister relationship
Experimental Methods				
Interval of experiment	About one-week interval	About one-week or two-weeks interval	Same as left	About two-weeks inter
Administration period of pesticide	From July 9, 2011 to the colony extinction, or to December 3 (147 days)	From July 21, 2012 to the colony extinction, or to August 16 (26 days)	From September 5, 2013 to the colony extinction, or to December 1 (87 days)	From July 1, 2018 to the colony extinction
Starting time of each observation	Just after dawn if possible (before bees go out to forage)	Same as the left	Same as the left	Same as the left
Vehicle to administer a pesticide	Either sugar Syrup or pollen paste	Sugar syrup	Sugar syrup	pollen paste
Administration method of pesticide	A pesticide was dissolved in sugar syrup or pollen was kneaded with toxic sugar syrup containing the pesticide. Either toxic sugar syrup or toxic pollen paste was fed into a hive	A pesticide was dissolved in sugar syrup with toxic sugar syrup containing the pesticide. Only toxic sugar syrup was fed into a hive.	A pesticide was dissolved in sugar syrup with toxic sugar syrup containing the pesticide. Only toxic sugar syrup was continuously fed into a hive with an auto-feeding system composed of 10 L (14 kg syrup) container	Pollen paste was made while pollen was being kneaded with toxic sugar syrup containing the pesticide. Toxic pollen paste was fed into a hive.
Counting method of the number of adult bees	Directly counted with accuracy from photos of combs with bees and bees left in a hive after every comb was removed from it with the help of a automatic counting software	Same as the left	Same as the left	Same as the left
Counting method of the number of capped brood	Accurately counted from photos of combs without bees after shaking the bees off each comb	Same as the left	Same as the left	Same as the left
Total intake of pesticide per colony	Calculated from the sugar syrup or pollen paste with pesticide consumed by honeybees.	Calculated from the sugar syrup with pesticide consumed by honeybees	Same as the left	Same as the left
Estimation of the intake of pesticide per bee	Estimated from dividing the total intake of pesticide per colony by the total number of initial & newly-emerged honeybees	Same as the left	Same as the left	Same as the left
Counting method of number of dead bees	Dead bees were accurately counted one by one inside and outside a hive which was placed on a large tray	Same as the left	Same as the left	Same as the left
Confirmation and record methods of a queen	A photographic record of the existence of a queen in each colony	Same as the left	Same as the left	Same as the left
Publications of Research Results (Title, Journal, Short Abstract)				
Experimental Results	A clear difference in the impact on honeybee (<i>Apis mellifera</i>) colony between the two vehicles of sugar syrup and pollen paste T. Yamada et al. (2018a). <i>J. Biol. Ser.</i> 1(3): 084-107.	Difference between the impact of the neonicotinoid dinotefuran and organophosphate fenitrothion on a bee colony in a long-term field experiment: An evidence Yamada et al. (2018b). <i>J. Biol. Ser.</i> 1(3): 108-137	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) T. Yamada et al. (2018d). <i>J. Biol. Ser.</i> 1(4): 187-207.	
	This study investigates the difference in the impact of the neonicotinoid pesticide dinotefuran on honeybee 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.	We conducted a long-term field experiment and found different impacts on honeybee colonies in an apiary between the neonicotinoid dinotefuran and the organophosphate fenitrothion. The colony where dinotefuran was administered (dinotefuran colony) became extinct in the administration period of 26 days while the colony where fenitrothion was administered (fenitrothion colony) survived long after the same administration period. The fenitrothion colony succeeded in overwintering and staying alive for more than 293 days after administration. We speculate that colonies exposed to dinotefuran hardly recover from the damage because dinotefuran has a much longer persistent ability than fenitrothion and toxic foods stored in cells over a prolonged period of time can affect a colony.	In this paper the differences are investigated between neonicotinoids (dinotefuran, clothianidin) and organophosphates (fenitrothion, malathion) on a realistic pesticide-concentration level in the natural environment surrounding an apiary in Japan. It has been 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 because of their high degradability. These results roughly reproduced the findings in our previous experiment. Neonicotinoid-concentration in residual honey in comb-cells were lower than those in sugar syrup fed to each colony were detected but organophosphates were hardly detected. We 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	
Estimation results of Apparent longevity	A mathematical model to estimate the seasonal change in apparent longevity of bee colony			
	Y. Yamada et al. (2019). <i>Scientific Reports</i> , volume 9, Article number: 4102 (2019)			
	We have proposed a new mathematical model to estimate the apparent longevity defined in the upper limit of an integral equation. The apparent longevity can be determined only from the numbers of adult bees and capped brood. By applying the mathematical model to a honeybee colony in Japan, seasonal changes in apparent longevity were estimated in three long-term field experiments. Three apparent longivities showed very similar season-changes to one another, increasing from early autumn, reaching a maximum at the end of overwintering and falling approximately plumb down after overwintering.			
	Seasonal change in apparent longevity of a pesticide-exposed colony in mid-west of Japan			
T. Yamada et al., preparing paper submission (this work)				
Using our proposed the mathematical model to estimate the apparent longevity of a honeybee colony, we clarified the differences of the seasonal change in apparent longevity between pesticide-free colonies and colonies exposed to pesticides (neonicotinoids and organophosphates) and between the two vehicles of sugar syrup and pollen paste used to administer a pesticide. We observed a glaring difference in seasonal apparent longevity between the two vehicles: the pollen-paste-vehicle colony remained nearly constant in apparent longevity through the seasons, even during overwintering, while the other colonies show a well-known rapid increase in apparent longevity during overwintering.				

Supplementary Table S2. Weather information & Experimental time.

Date	Elapsed days	Weather	Temp.	Humidity	Experimental time		Remarks
	[day]		°C	%	from	until	
19-Jun-18	0	fine	24	65	6:00	8:30	Start of experiment
1-Jul-18	12	fine			5:35	8:00	Start of pesticide administration
16-Jul-18	27	fine	25	76	5:30	7:50	
28-Jul-18	39	cloudy	23	71	5:30	7:40	
12-Aug-18	54	fine	22	73	5:30	7:31	
28-Aug-18	70	cloudy → rainy	25	83	5:30	7:40	
11-Sep-18	84	fine	19	93	5:30	7:30	
23-Sep-18	96	fine	16	79	5:30	7:30	
6-Oct-18	109	fine	20	84	6:00	7:50	
23-Oct-18	126	fine	10	79	6:00	7:40	DF-3 became extinct.
4-Nov-18	138	fine	9	87	6:15	7:50	
18-Nov-18	152	fine	5	82	6:30	8:00	
2-Dec-18	166	fine	-0.2	75	6:45	7:50	CR-2, CR-3, DF-1 and DF-2 became extinct.
16-Dec-18	180	fine	-1	77	8:00	8:15	DF-1 became extinct. Finish of experiment

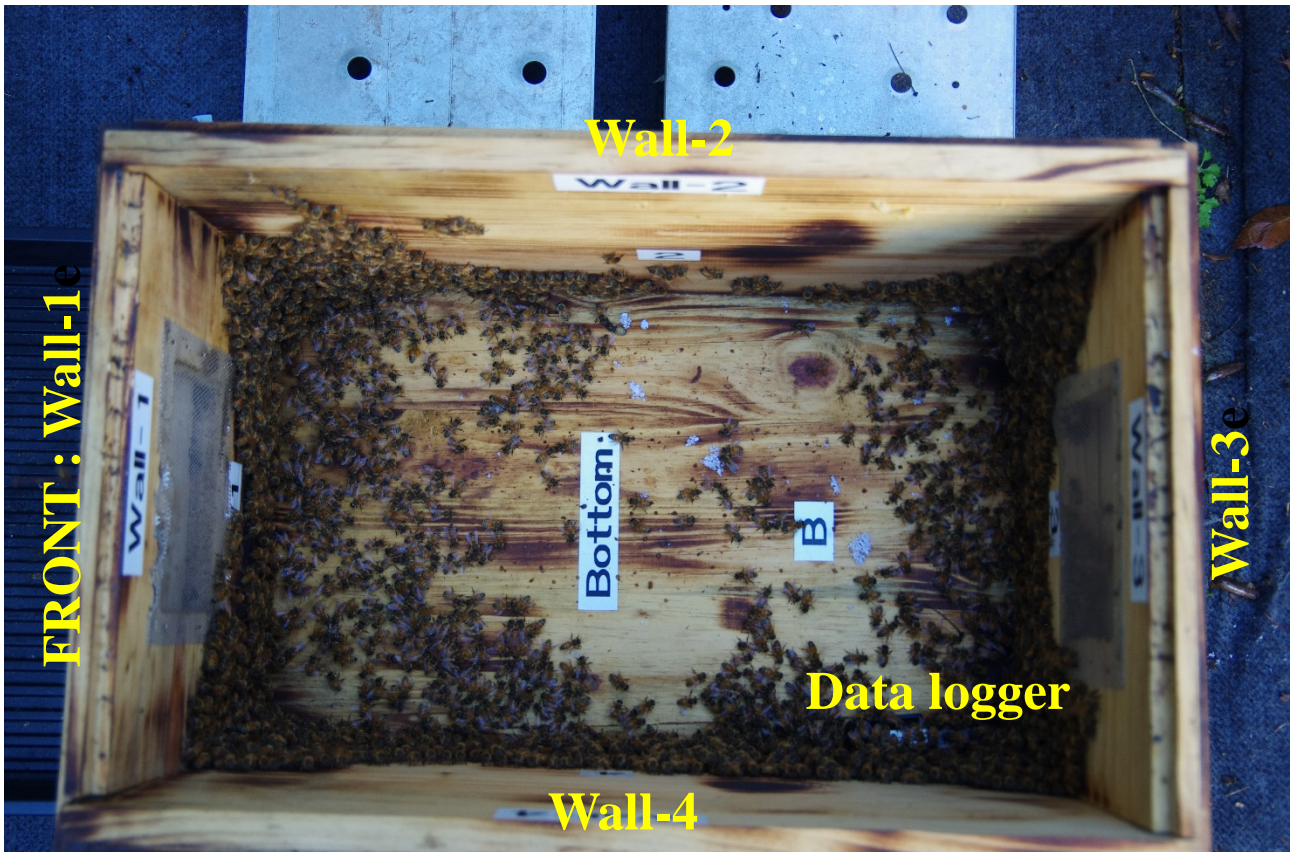
Supplementary Table S5. Summary of experimental note.

“Prep.” and “Obs.” are abbreviations for preparation and observation, respectively. **Bold** sentences are the notable points.

Date	Elapsed days from exp start [day]	Elapsed days from pesticide admin. [day]	Kind of work	Contents
9-Jun-18	-10	-22	Prep.	Land preparation and mowing around experimental site as were carried out for field experiment.
14-Jun-18	-5	-17	Prep.	Eight bee colonies with the sister queen were purchased from Shitahashi Bee Farm and They ere moved from kanazawa city to Shika (about 70 km distance) town over 1.5 hours. Stickers (lavels) for explanation were attached on each comb frame (continues) and each new hive box (finished).
15-Jun-18	-4	-16	Prep.	Stickers (lavels) for explanation were attached on each comb frame (continues) and each new hive box. Pesticide-free pollen paste and sugar syrup were fed to all colonies for dietary supplement to the colonies. Honeybees in old hive boxe were replaced with new hive boxes.
16-Jun-18	-3	-15	Prep.	Stickers for explanation were attached on each comb frame (finished). Six colonies were choosed from eight colonies. Six colonies were placed with their front facing south and west to east facing the front.
18-Jun-18	-1	-13	Prep.	Data loggers (EL-USB2) were put on the bottom of the hive boxes of CR-1, CR-3, DF-1 and DF-3 to measure temperatures and humidity in the hive boxes and under the hive box of DF-2 to measure them in the ambient ones.
19-Jun-18	0	0	Obs.	Field experiment was started. Extra combs were made outside the bottom of each container to feed sugar syrup. Pesticide-free pollen paste and sugar syrup were fed to all the colonies.
23-Jun-18	4	-8	Prep.	Preparation of pollen paste and sugar syrup : Using 20kg of sugar and 13.33kg of water, 33.33kg of 60% sugar syrup was made. Using 7.8 kg of pollen and 5.2 kg of sugar syrup, 13 kg of 60 % pesticide-free pollen paste was made. Using 6 kg of pollen and 4 kg of sugar syrup containing 40g of 100ppm dinotefuran (DF) sugar-syrup, 10 kg of 60 % pollen paste was made. The amounts of pollen paste are 10 doses to each colony. Pollen paste with DF was packed in 300g trays and stored in the refrigerator freezer. Pesticide-free pollen paste was packed in 300g trays and stored in the refrigerator's refrigerator compartment.
1-Jul-18	12	0	Obs.	Start of pesticide administration : 300 g of pollen paste of with dinotefuran 0.4 ppm was first fed to DF-1, D-2 and DF-3 only. Pesticide-free pollen paste was fed to all control colonies of CR-1, CR-2 and CR-3. 800 g of pesticide-free sugar syrup was fed to all colonies. The queen bee could not be found in DF-3and there were many queen cells in it. The queen bee in the auxiliary colony was moved to DF-3 after being put in a queen cage. The queen bee was released from the queen cage after about 9 hours. We seemed to be fine at first glance, but would check the new ueen bee again the next day. All the queen cells in DF-3 were removed from combs and only six queen cells in them were put in the auxiliary colony.
2-Jul-18	13	1	Others	The new queen bee in DF-3 was accepted by honeybees in DF-3 without any problems.□
16-Jul-18	27	15	Obs.	Extra combs were made on the division board in each hive box. An additional comb frame (NO.5) was added in CR-1, CR-2 and CR-3 because they seemed to be strong (many honeybees). The DF-administered colonies also seemed to be strong because they would be weakened by the pesticide of dintefuran.
10-Aug-18	52	40	Prep.	Preparation of pollen paste and sugar syrup : Using 20kg of sugar and 13.33kg of water, 33.33kg of 60% sugar syrup was made. Using 2.4 kg of pollen and 1.6 kg of sugar syrup, 4kg of 60 % pesticide-free pollen paste was made. Using 2.4 kg of pollen and 1.6 kg of sugar syrup containing 24g of 100ppm dinotefuran (DF) sugar-syrup, 4 kg of 60 % pollen paste was made. The amounts of pollen paste are 6 doses to each colony. Pollen paste with DF was packed in 300g trays and stored in the refrigerator freezer. Pesticide-free pollen paste was packed in 300g trays and stored in the refrigerator's refrigerator compartment.
12-Aug-18	54	42	Obs.	New addition of DF pollen paste : All clonies seemed to be strong. As DF-administered colonie became very large, it was assumed that the amount of pesticide intake per beel was too small with the conventional dose. We tried to increase the amount of pesticide administered to the DF-administered colonies by the addition of 200g pollen paste with 0.6 ppm DF to 300 g pollen paste with 0.4 ppm for DF-1, DF-2 and DF-3. Pesticide-free pollen paste of 200g also was added into CR-1, CR-2 and CR-3. Later I found that this increasement of toxic pollen paste in DF colonies, was not well suited.
28-Aug-18	70	58	Obs.	A hornet capture was installed at the front of each hive box. The experiment was executed in the rain.
7-Sep-18	80	68	Others	Attacks by Japanese giant hornets (<i>V. m. japonica</i>) : The hornet capturer that was installed and the corrugated roofs set on the hive box was all removed by Typhoon No. 21. CR-2 and DF-1 were greatly damaged by the Japanese giat hornets. Number of dead bees were 1675 on the tray of CR-2 and 347 on the tray of DF-1.. A few dead Japanese giat hornets were found around each hive box: 3 (DF-1); 4 (CR-2); 2 (DF-2).
11-Sep-18	84	72	Obs.	Pollen paste was left over in DF-1, DF-2 and DF-3. Honeybees in DF-1, DF-2 and DF-3 looked frustrated though those in CR-1, CR-2 and CR-3 looked calm. We found a great number of dead bees in the hive box of DF-3. Though the queen bee was found in all hive boxes, the queen cells was found in DF-1.
23-Sep-18	96	84	Obs.	Honeybees in DF-1, DF-2 and DF-3 looked restless. The amount of pollen paste was put back from 500g to 300g; from 300g of 0.4 ppm pollen paste and 200g of 0.6 ppm one to 300g of 0.4ppm one in each DF-administeed colony (DF-1, DF-2, DF-3). Wax-worms were found DF-3.
6-Oct-18	109	97	Obs.	Diarrhea stools were found in DF-1 and DF-2. Wax-worms were found in CR-2 and DF-1. In DF-1 and DF-3 the queen bee was absent and the number of honeybees wa svery small. Especially, DF-3 was on the verge of extinction.
23-Oct-18	126	114	Obs.	DF-3 became extinct. DF-1, in which the queen bee was absent and which seemed to have chalkbrood disease, was on the verge of extinction. Wax-worms were found in DF-1 and DF-3.
4-Nov-18	138	126	Obs.	CR-1, CR-3 and DF-1 became weak. Wax-worm were found in CR-2. DF-1 had no queen bee.
18-Nov-18	152	140	Obs.	All colonies became weak. DF-1 and DF-2 had no queen bee (A dead queen bee was found at the bottom of DF-2). To prepare for overwintering, the hive boxes were covered with foamed polystyrene boards. Many Varroa mites (Varroa destructor; Varroa jacobsoni) were found in DF-2.
2-Dec-18	166	154	Obs.	CR-2, CR-3, DF-1 and DF-2 became extinct. The queen bee in CR-1 became cold and deadly when the hive box was open for observation. Dead queen bees were found in CR-2 and CR-3. Disposable handy warmer sachet (Hokkairo) was added in CR-1 to warm the colony. Overwintering work was completed.
16-Dec-18	180	168	Obs.	DR-1 became extinct. All colonies became extinct. Finish of experiment.

Supplementary Figure S1. The inside of the hive box is numbered clockwise from the face of the entrance.

In order to record the state of the inside of the hive-box in the photograph, the wall number clockwise from the entrance is attached to the wall of the inside of the hive-box, and the bottom of the nest box is also specified. The temperature and humidity data logger “EasyLog/EI-USB-2” was placed inside CR-1, CR-3, DF-1, DF-3 and under the DF-2 hive-box (in the tray) to achieve the outside (ambient) temperature near the hive-box.



Supplementary Figure S2. An example of numbered comb frame.

Information identifying the comb frame in the hive-box is clearly stated on the comb frame. This photo image will be described as an example. "3F" identifies the position and surface of the nest monument frame in the hive-box, in this example, it shows the left side of the third comb frame from the left toward the front of the hive-box. "CR-1" of "CR-1-3F" shows the control group of CR-1, and "3F", as described above, represents the position of the comb frame in the hive-box.



Supplementary Figure S3. Photo stand, two empty hive-boxes, experiment log and beekeeping tools.

In order to prevent vibration to the bee at the time of photography, and to keep the comb frame calm, I made my own photo stand. The comb frame with the bee is pulled out from the hive-box, and after taking the photograph of both sides of the comb frame, once, in order to store the comb frame with the bee, two empty hive-boxes are prepared in advance.



Supplementary Figure S4. Overall view of experimental site.

Each hive-box was arranged in the order of CR-1, DF-1, CR-2, DF-2, CR-3 and DF-3 from west to east every 80 cm, with the entrance facing south. Incidentally, CR-1, CR-2 and CR-3 are the control (pesticide-free) colonies and DF-1, DF-2 and DF-3 are the dinotefuran-exposed colonies where 0.4 ppm of dinotefuran are administered via pollen paste.

