Journal Biological Series 1(3): 108-137, July 2018
DOI: 10.15413/ajbs2018.0401


Academia Publishing

*Research Paper*

# Difference between the impact of the neonicotinoid dinotefuran and organophosphate fenitrothion on a bee colony in a long-term field experiment: An evidence

*Accepted 9th August, 2018*

**ABSTRACT**

Neonicotinoides are long-persistent and highly toxic pesticides that have become popular instead of organophosphates and are strongly suspected to cause massive disappearance of bees. On the other hand, the evaluation of a long-term influence of a pesticide on a honeybee colony in the natural environment is not yet established. In this paper, we conducted a long-term field experiment and found different impacts on honeybee colonies (*Apis mellifera*) in an apiary between the neonicotinoid dinotefuran and the organophosphate fenitrothion. Each concentration of the pesticides in sugar syrup provided for honeybees was adjusted at the same insecticidal activity to exterminate stinkbugs. The colony where dinotefran 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, which seems to be able to recover even after the exposure to fenitrothion. The dinotefuran colony became extinct though the intake of dinotefuran was estimated to be comparable with that of fenitrothion in terms of the $LD_{50}$ to a honeybee. Judging from the results in this work and our previous works, 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.

**Key words:** Dinotefuran, neonicotinoid, fenitrothion, organophosphate, CCD, sugar syrup, field experiment, long-term, pesticide, honeybee, colony, overwintering, colony distinction, acute toxicity, chronic toxicity, *Apis mellifera*.

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## INTRODUCTION

Massive losses of honeybee colonies are becoming a worldwide problem (van Engelsdorp et al., 2011; van Engelsdorp et al., 2012; Spleen et al., 2013; Steinhauer et al., 2014; van der Zee et al., 2012, 2014; Pirk et al., 2014). Many researchers in trying to find out why honeybee colonies are becoming a worldwide problem have proposed various causes such as pesticides, mites, pathogens and their hybrids. Recently pesticides, especially neonicotinoid pesticides (neonicotinoids) which are persistent, systemic and highly neurotoxic, are strongly suspected of causing massive losses based on many laboratory experiments and several long-term field experiments (van der Sluijs, 2013). Neonicotinoids have been widely used in the world at present, even after a moratorium in the EU on the use of three neonicotinoids (imidacloprid, clothianidin and thiamethoxan) under the given limitations. In 2013, many papers reported on the adverse effects of neonicotinoids on insects (Prisco, 2013; EFSA, 2013a, b, c, d; Hatjina et al.,

2013; Hunt and Krupke, 2013), mammals (EFSA, 2013e; Bal et al., 2013) and humans (Taira et al., 2013).

A neonicotinoid was evaluated by the $LD_{50}$ (50% lethal dose) which is one way to measure the short-term poisoning potential (acute toxicity) of a material. This value can give the useful information on the acute toxicity in a short-term dose but cannot evaluate the chronic toxicity in a long-term. In order to elucidate the anomalous behaviors of honeybee colony such as a colony collapse disorder and failure in wintering, the illuminating assessment of the impact of chronic toxicity on a honeybee colony in the fields will be more significant than that of acute toxicity.

Field experiments on a honeybee colony are affected by many uncontrollable factors such as honeybee behavior, weather, vermin (hornets, mites and hive-beetles,) and pathogens etc. Supposing that field experiments are conducted under the same environmental conditions for all the colonies, it becomes important to evaluate the honeybee behavior of an experimental colony because the environmental factors other than honeybee behavior will be largely offset by a control colony. The honeybee behavior such as foraging, nursing, ovipositing, reproducing, storing and repairing etc is greatly affected by pesticides. The uncontrollable behaviors of honeybees are mutually related and interact to each other as eusocial insects in the fields. Therefore, the experimental results in a controllable laboratory test under certain limited and special circumstances cannot be always applied to those in field testing. In addition to this, when comparing the $LD_{50}$ with the pesticide amount ingested by an experimental colony in field testing, it should be considered that honeybees may prefer pesticide-free nectar and natural pollen to sugar syrup and artificial pollen containing a pesticide.

It was confirmed from our previous works (Yamada et al., 2012, 2018), that the following adverse effect of a neonicotinoid pesticide on a honeybee colony: High-concentration neonicotinoid in sugar syrup (10 ppm of dinotefuran and 4 ppm of clothianidin) and pollen paste which is made after mixing them with pollen, collapsed the honeybee colonies due to the acute toxicity: Low-concentration neonicotinoids (1 ppm of dinotefuran and 0.4 ppm of clothianidin) collapsed the colonies due to the chronic toxicity after having assumed the appearance of a Colony Collapse Disorder (CCD) or failure in wintering: Middle-concentrations neonicotinoids (dinotefuran of 2 ppm and clothianidin of 0.8 ppm) caused damage to a colony due to acute toxicity just after the start of pesticide administration and due to the chronic toxicity at the later period after having assumed the appearance of a CCD which finally collapsed them.

It was confirmed that honeybees ingest toxic foods (sugar syrup and pollen paste) in the beehive even when they could freely take non-toxic nectar from fields. Even though the low-concentration pesticides in our previous studies could cause instantaneous death of honeybees due to acute toxicity judging from the $LD_{50}$, in actual fact, they hardly caused any instantaneous death. This result is thought to be attributed to the dilution of toxic sugar syrup or pollen paste containing a pesticide administered into a beehive by non-toxic nectar or natural pollen gathered from the pesticide-free fields. The dilution ratio of toxic sugar syrup or pollen paste by pesticide-free nectar or natural pollen selectively taken from fields depends greatly on the weather which affects the foraging activity of honeybees. These suggest that it is quite inadequate to assume that the field experimental conditions can be determined from the results of laboratory testing which is conducted under controlled conditions.

Recently, Pilling et al. (2013) reported that no detrimental effects on colony survival and overwintering success was found at four-year repeated field exposures of thiamethoxam (1 to 7 ppb) to pollen and nectar. However, the experimental concentrations of thiamethoxam are much lower than the residue concentration (53 ppb in pollen) as reported by Johnson et al. (2010) and can be probably too low to affect a colony even due to its chronic toxicity.

Considering the perils to the environment of pesticides whose concentrations changes with time, the impact of a pesticide at higher environmental concentrations on honeybee colonies should be discussed because the maximum environmental concentration pesticide would cause serious damage to honeybee colonies and a very low concentration pesticide can hardly affect honeybee colonies due to their excretion after little absorption. Incidentally, the actual average year-round concentration of a pesticide included in stored honey on a frame of comb (hereafter, comb) is unclear and the cumulative total intake of pesticide per bee unknown as reported by Pilling et al. (2013). Further, the result by Pilling et al. (2013) may be attributable to the dilution of poisoning pollen and nectar fed to a colony with non-toxic ones from fields, or only a very slight intake of toxic honey or pollen fed to a colony by honeybees.

Yamada et al. (2012) conducted the field experiment at low, middle and high concentrations of neonicotinoids (dinotefuran and clothianidin) and Yamada et al. (2018) at low and high concentrations of dinotefuran. So far, low- and high- concentration field-experiments of dinotefuran have been conducted twice (Yamada et al., 2012, 2018) whose results have been replicated respectively but the middle one has been done only once (Yamada et al., 2012). Our previous results (Yamada et al., 2012) revealed that through a long-term field experiment neonicotinoids lead to gradual extinction of a honeybee colony due to chronic toxicity after the occurrence of many instantaneous honeybee-deaths at high concentrations; some instantaneous honeybee-deaths at middle concentration and no instantaneous honeybee-deaths at low concentration is due to acute toxicity in the beginning of the experiment. The honeybee colony, which was exposed to neonicotinoids and escaped early extinction due to acute toxicity dwindled away to nothing as a result of chronic toxicity after showing an aspect of CCD or failed overwintering.

Dinotefuran and fenitrothion are known as representative

pesticides of neonicotinoids and organophosphates in Japan, respectively. We demonstrated that a neonicotinoid pesticide such as dinotefuran and clothianidin causes an aspect of CCD before the extinction of honeybee colony as reported by Yamada et al. (2012). Now the question remains whether organophosphate pesticides, which were popular before the appearance of neonicotinoids cause a CCD. Though the impact of fenitrothion on birds, insects, fish, honeybees and the persistent residues in the environment has been widely investigated by long-term field monitoring (Mitchelland, 1984), it is uncertain whether fenitorothion causes a CCD or not. In this work we will confirm whether the results obtained for neonicotinoids in our previous works (Yamada et al., 2012, 2018) can be applied to organophosphates such as fenitrothion or not. Here, we will elucidate the impact of fenitrothion on a honeybee colony during long-term exposure to a pesticide comparing it with dinotefuran. In this work we will clarify the followings: (1) Which pesticide will cause a honeybee colony to become extinct faster in a situation where each pesticide concentration in sugar syrup is prepared to be identical in insecticidal activity for stinkbugs, the neonicotinoid dinotefuran or the organophosphate fenitrothion? Which has actually higher toxicity for a honeybee colony? (2) How will each colony behave when we feed toxic sugar syrup which is newly prepared? What difference in behavior of a honeybee colony can be caused by dinotefuran and fenitrothion? (3) How will the surviving colony behave after it is damaged by the pesticide when it continues to take non-toxic sugar syrup instead of toxic sugar syrup just after either colony has become extinct? How much longer will the stored toxic sugar syrup (honey) in the beehive continue to affect the honeybee colony?

## MATERIALS AND METHODS

### Materials and preparation of pesticide concentrations

Table 1 shows the experiments were performed from 2012 to 2013 under experimental conditions. STARCKLE MATE (10% dinotefuran; Mitsui Chemicals Aglo, Inc., Tokyo, Japan) and SUMITHION EMULSION (50% fenitrothion; Sumitomo Co. Ltd., Osaka, Japan) were used in this study. In order to compare the effects on honeybees between dinotefuran and fenitrothion, the concentration of each pesticide was prepared so as to be identical in insecticidal activity to exterminate stinkbugs in general usage in Japan where the insecticidal activity was virtually synonymous with the short-term effect on a honeybees such as the median lethal dose ($LD_{50}$) in this case. Each concentration of dinotefuran and fenitrothion was determined at the one-fiftieth of the spraying concentration (100 ppm for dinotefuran and 500 ppm for fenitrothion) to exterminate stinkbugs by referring to our previous results, which was 2 ppm of dinotefuran (called “Middle” concentration in Yamada et al., 2012) and fenitrothion of 10 ppm, respectively. Neonicotinoids of dinotefuran and clothianidin, which are adjusted in concentration, have the same insecticidal activity affecting stinkbug, which are confirmed to have almost the same effect on honeybees. The middle concentrations of dinotefuran and clothianidin resulted in some instant honeybee-deaths at the beginning and afterwards the gradual extinction of a honeybee colony after giving the appearance of a CCD when they are administered into colonies through both sugar syrup and pollen paste (Yamada et al., 2012).

Incidentally, focusing on the $LD_{50}$, the $LD_{50}$ values of dinotefuran and clothianidin widely ranges from 7.6 ng/bee (US-EPA, 2004) to 75 ng/bee (Iwasa et al., 2004) and from 20 ng/bee (US-EPA, 1995) to 380 ng/bee (US-EPA, 1995), respectively. The average of the minimum and maximum values of each $LD_{50}$ is about 41 ng/bee for dinotefuran and 200 ng/bee for fenitrothion, respectively. Judging from the ratio of the average of fenitrothion to that of dinotefuran which is about five, 2 ppm of dinotefuran and 10 ppm of fenitrothion, which have the same insecticidal activity to exterminate stinkbugs, can be estimated to have almost the same insecticidal activity in terms of the $LD_{50}$ for a honeybee.

As the frequency of spraying of a pesticide (dinotefuran, fenitrothion) is usually three or four times in order to exterminate stinkbugs in rice cropping in Japan, we have determined to administer fresh pesticides newly prepared four times. We observed the colonies and got a photographic record of their states; all combs with and without honeybees, and the inside of a beehive and the outside just before the time when fresh toxic sugar syrup with pesticide is administered (hereafter, administration date) and the day after in order to investigate an acute toxic effect of insecticidal activity of a pesticide. Comparing the numbers of adult bees and dead bees the day after the administration date with those numbers on the observation date after about one week, we conjectured the change in toxicity of the administered pesticide. Fresh toxic sugar syrup containing a newly-prepared pesticide continues to be fed into a beehive (colony) after the administration date until it is replaced with fresh toxic sugar syrup on the next administration date. The administration date is virtually synonymous with the observation date in this work, but fresh toxic sugar syrup is not always fed to a colony on every observation date. The administration date means the date when fresh toxic sugar syrup containing a newly prepared pesticide is fed into a beehive (colony) through a feeder after an already-existing feeder with old toxic sugar syrup has been replaced with a new feeder with fresh toxic sugar syrup. This indicates the date when fresh toxic sugar syrup contains a newly-prepared pesticide. Thereafter, the observation date means the date when we observationally conduct an experiment in which we take photographs of the states of each colony (every combs both with and without adult bees, adult bees on four inside walls and an inside bottom, a queen, signs of disease, vermin and evidences of attacks by Asian giant

**Table 1:** Outline of experimental conditions in this work.

| **Experimental objective** | **Experimental contents** |
|---|---|
| | **Difference between neonicotinoid and organophosphate pesticides** |
| Kind of pesticide | dinotefuran (STARCKLEMATE 10®); fenitrothion (SUMITHION emulsion®) |
| Experimental period | From June 28th 2012 (Acclimatization period of a colony: June 28th to July 21st ) to May 10th in 2013 (Observations were continued till July 14th 2013). |
| Pesticide administration period | From July 21st 2012 till any colony has become extinct (the dinotefuran-dosage colony has become extinct on August 16th 2012 earlier than the fenitrothion-dosage colony) |
| Vehicle (food) to administer a pesticide to a colony | Sugar syrup |
| Concentration of pesticide in sugar syrup | 2 ppm (dinotefuran); 10 ppm (fenitrothion) |
| Frequency of administration of fresh pesticide newly prepared | Three times (the spray frequency of a pesticide in rice cropping in Japan) |
| Number of colony | Four colonies: Two controls (RUN1 and RUN4) which were arranged at the southern end and at the northern end because of the offset of position influence ; two experimental colonies which were exposed to dinotefuran (RUN 2) and to fenitrothion (RUN3) |
| Circumstances in an apiary | No crop-dusting within 2 km around, establishment of a new pesticide-free watering place and new plantings of honey crop without the exposure to pesticides in the apiary for experiments |
| Number of two tiered hive box | Four hives (two controls and two dose tests) |
| Kind of honeybees | *Apis mellifera* |
| Initial composition of a hive | Three combs with full bees and some brood and an auto-feeding system with a tank of 10 L (sugar syrup=14 kg) newly made for this experimental use as shown in Figure 1 |
| Initial number of honeybees and brood at the start of pesticide-administration | Both were about 10,000. |
| Frequency of observation | At intervals of about one week (When we administered newly-prepared sugar syrup with a pesticide to a colony, we observed all colonies and recorded their conditions by photos on the administration day and the day after) |
| Record of colony conditions | Photos of all combs and the inside of a hive with honeybees and all combs without honeybees taken in every observation |
| Number of adult bees in a hive | Directly counted with photos of all combs and the inside of a hive one by one after image processing with "Perfect Viewer 7" made by Nanosystem Corporation, Japan |
| Number of brood in a hive | Directly counted with photos of all combs without honeybees after image processing with "Perfect Viewer 7" made by Nanosystem Corporation, Japan |
| Number of dead bees | Directly counted in and around a hive one by one with tweezers |
| Intake of pesticide of honeybees | Accurately weighed by a weighing instrument at the end of experiment |
| Administration method of pesticide | Administration of toxic sugar by an auto-feeder with 10L-tank (sugar syrup=14 kg) storing them in each hive |
| Prevention of swarming | Experiment start after the swarming period |
| Confirmation of a queen bee | Record by photos |
| Water feeding site | Provide water feeding site in the apiary |
| Hornet catcher | Installation of a hornet catcher in each hive after summer |
| Starting time of each experiment | Early morning except rainy day because of the prevention of a decrease in number due to foraging |
| Others | Record by photos about troubles such as wax worms, bee-beetles, etc. |

hornets), the vicinity of each beehive, and record our observations of what occurred in the experiment and newly put a feeder filled with fresh toxic sugar syrup in a beehive after weighing the amount of toxic sugar syrup remaining in a feeder and thereafter emptying out the remaining toxic sugar syrup from the feeder.

The experimental concentrations of these pesticides (2 ppm of dinotefuran and 10 ppm of fenitrothion which will have the same insecticidal activity for stinkbugs as 2 ppm of dinotefuran) seem to be realistic and possible in the field of Japan, judging from the facts that the concentration of clothianidin near rice paddies was about 5 ppm (Kakuta et al., 2011) where the insecticidal activity of clothianidin to honeybees was about 2.5 times that of dinotefuran (Yamada et al., 2012) and maximum residue limits (MRLs) of dinotefuran in foods in Japan (JFCRF, 2017) are 2 ppm for rice (brown rice), 50 ppm for tea, 2 ppm for Chinese cabbage, 10 ppm for Japanese mustard spinach, 25 ppm for lettuce, 15 ppm for spinach, 2 ppm for tomato, 2 ppm for apple, 15 ppm for grape and 10 ppm for orange etc (Table 2).

**Methods used in field experiments**

Four beehives, each with 4 numbered combs (frames) and a feeder were sited facing east on a hill. They were aligned in order of RUN number; the control colony (RUN1), the dinotefuran-dosage (RUN2), the fenitrothion-dosage (RUN3) and the control (RUN4) from the south to the north. Two controls were arranged at both ends because of the confirmation of difference between the north and south.

Pesticide-free sugar syrup was fed into every colony from June 28$^{th}$ 2012 to the early morning of July 21$^{st}$ as a preliminary experiment in order to acclimatize the colonies to the experimental apiary after the swarming season. After the period of acclimatization, we administered each pesticide into the dinotefuran colony (RUN2) and the fenitrothion colony (RUN3), respectively, till either colony became extinct while each toxic sugar syrup with a pesticide (fenitrothion or dinotefuran) was replaced with newly-prepared (fresh) toxic sugar syrup every administration date after each residual quantity of toxic sugar syrup in a feeder was weighed with an accuracy down to units of 0.1 g. After the four administrations of each fresh toxic sugar syrup, only the dinotefuran colony became extinct. After one experimental colony (dinotefuran colony) became extinct, we exchanged toxic sugar syrup with pesticide-free sugar syrup in the other surviving experimental colony (fenitrothion colony) in order to investigate whether the surviving colony exposed to the pesticide can recover from the damage of the pesticide or not under the pesticide-free conditions.

We observed all colonies and took photos of all combs with bees, those without bees, the inside with residual bees of each beehive (four walls and bottom of the beehive where there is no comb and no feeder, a queen, queen-cells, evidences of honeybee diseases such as chalk brood and giant-hornet attacks and surrounding circumstances about every week on the administration date and the day after). The number of adult bees on all combs, which were numbered and ordered numerically in every beehive, and a feeder and the inside of the beehive (4 walls and bottom) was counted over again directly and accurately from photographs (sometimes enlarged) of all combs while making a visual identification after the number of adult bees on each photograph was roughly counted with the aid of "Perfect Viewer 7" made by Nanosystem Corporation, Japan. Figure 1 shows the sample images when counting adult bees on comb, the remaining adult bees in the beehive and capped brood after directly shaking the bees off each comb. Though we have tried to develop and improve a new automatic counting software "Perfect Viewer 7" with binarizing photo images, we cannot always succeed in accurate counting of them because it cannot accurately count overlaid bees, bees and capped brood on a blurred images, those on a low contrast image or on a low brightness image even when changing and optimizing the threshold. Therefore, the software was used as an auxiliary to count adult bees and capped brood. To obtain the number of dead bees around the beehive, the beehive was placed on a large tray with many small weep-holes. The number of dead bees in the tray, beehive and feeder was counted directly, one after the other with a pair of tweezers.

The queen bee in the beehive was photographically recorded on each observation date as specific situations such as the presence of chalk brood or wax moth larvae and the evidence of Asian giant hornet attacks. In addition to taking photographs, the aspect of each beehive continued to be recorded at intervals of 1 h with a digital camera during the experimental period.

We performed the experiment early in the morning on fine or cloudy days before the foraging bees left the beehive from June 28$^{th}$ 2012 to May 10$^{th}$ 2013 (316 days). We observed the pesticide-free colonies till July 14$^{th}$ 2013 (381 days) after finishing this experiment (May 10$^{th}$ 2013) in order to clarify the normal behavioral standards of a honeybee colony for a year.

In order to decrease in unclearness and diversity of uncontrollable factors contained in field experiments, we selected an experimental site where there are no aerial-sprayed paddy fields and orchards in the vicinity. We located a honeybee-watering place in the experimental apiary to supply pesticide-free water and planted organically leaf mustard (*Brassica juncea*) and hairy vetch (*Vicia villosa*) in the experimental site to prevent honeybees from taking nectar and pollen contaminated by pesticides in order to minimize the effects of environmental factors.

The consumption of sugar syrup by honeybees was accurately measured by a weighing instrument having an accuracy of 0.1 g in every observation. The net intake of a pesticide was obtained from the amount of sugar syrup consumed by honeybees. The cumulative total intake of each

**Table 2:** Extracts from Maximum Residue Limits (MRLs) list of agricultural chemicals in foods in Japan.

| Foods | Maximum Residue Limits (MRLs) [ppm] Updated on July 19, 2017 | | | |
|---|---|---|---|---|
| | Acetamiprid | Clothianidin | Dinotefuran | Imidacloprid |
| Rice (brown rice) | 0.01 | 1.00 | 2.00 | 1.00 |
| Wheat | 0.30 | 0.02 | 0.01 | 0.20 |
| Cacao beans | 0.01 | 0.02 | 0.01 | 0.05 |
| Coffee beans | 0.01 | 0.05 | 0.01 | 0.70 |
| Hop | 0.01 | 0.10 | 0.01 | 7.00 |
| Tea | 0.01 | 25.00 | 50.00 | 10.00 |
| Asparagus | 0.50 | 0.70 | 0.50 | 0.70 |
| Broccoli | 2.00 | 1.00 | 2.00 | 5.00 |
| Cabbage | 3.00 | 0.70 | 2.00 | 0.50 |
| Cauliflower | 1.00 | 0.30 | 2.00 | 0.40 |
| Chinese cabbage | 0.50 | 2.00 | 2.00 | 0.50 |
| Japanese radish, roots (including radish) | 0.20 | 0.20 | 0.50 | 0.40 |
| Japanese radish, leaves (including radish) | 5.00 | 5.00 | 10.00 | 4.00 |
| KOMATSUNA (Japanese mustard spinach) | 5.00 | 10.00 | 10.00 | 5.00 |
| Lettuce (including cos lettuce and leaf lettuce) | 10.00 | 20.00 | 25.00 | 3.00 |
| Onion | 0.20 | 0.02 | 0.01 | 0.07 |
| SHUNGIKU | 10.00 | 10.00 | 20.00 | 3.00 |
| Spinach | 3.00 | 40.00 | 15.00 | 15.00 |
| Turnip, roots (including rutabaga) | 0.10 | 0.50 | 0.50 | 0.40 |
| Turnip, leaves (including rutabaga) | 5.00 | 40.00 | 5.00 | 3.00 |
| Cucumber (including gherkin) | 2.00 | 2.00 | 2.00 | 1.00 |
| Egg plant | 2.00 | 1.00 | 2.00 | 2.00 |
| Green soybeans | 3.00 | 2.00 | 2.00 | 3.00 |
| Strawberry | 3.00 | 0.70 | 2.00 | 0.40 |
| Tomato | 2.00 | 3.00 | 2.00 | 2.00 |
| Water melon | 0.30 | 0.20 | 0.50 | 0.50 |
| Apple | 2.00 | 1.00 | 2.00 | 0.50 |
| Banana | 0.01 | 1.00 | 0.01 | 0.04 |
| Grape | 5.00 | 5.00 | 15.00 | 3.00 |
| Grapefruit | 2.00 | 2.00 | 10.00 | 0.70 |
| Japanese pear | 2.00 | 1.00 | 1.00 | 0.70 |
| Japanese persimmon | 1.00 | 0.50 | 2.00 | 1.00 |
| Lemon | 2.00 | 2.00 | 10.00 | 0.70 |
| Orange (including navel orange) | 2.00 | 2.00 | 10.00 | 0.70 |
| Peach | 2.00 | 0.70 | 3.00 | 0.50 |
| UNSHU orange, pulp | 0.50 | 1.00 | 2.00 | 0.30 |
| Milk | 0.10 | 0.02 | 0.05 | 0.10 |
| Honey (including royal-jelly) | 0.20 | 0.01 | 0.01 | 0.01 |

**Note:** Uniform limit is 0.01 ppm.

active ingredient (dinotefuran and fenitrothion) was obtained from the amount of sugar syrup consumed by honeybee colony during the pesticide-administration period. The interval intake of a pesticide by a colony between two adjacent observation dates (a certain observation date and the previous one) was obtained from the consumption of sugar syrup with a pesticide. The intake of a pesticide per bee was estimated from dividing the cumulative total intake of the pesticide in a colony by the sum of the number of newborn honeybees, the number of initial honeybees and that of the capped brood at the colony extinction under the assumption that the capped brood at the colony extinction had already taken the pesticide.

Strictly speaking, this experiment cannot be always conducted under the very same conditions as the natural environment near an actual apiary, because sugar syrup is not same as nectar in fields and the feeding area in this work is not the same as that in an actual apiary. That is, honeybees

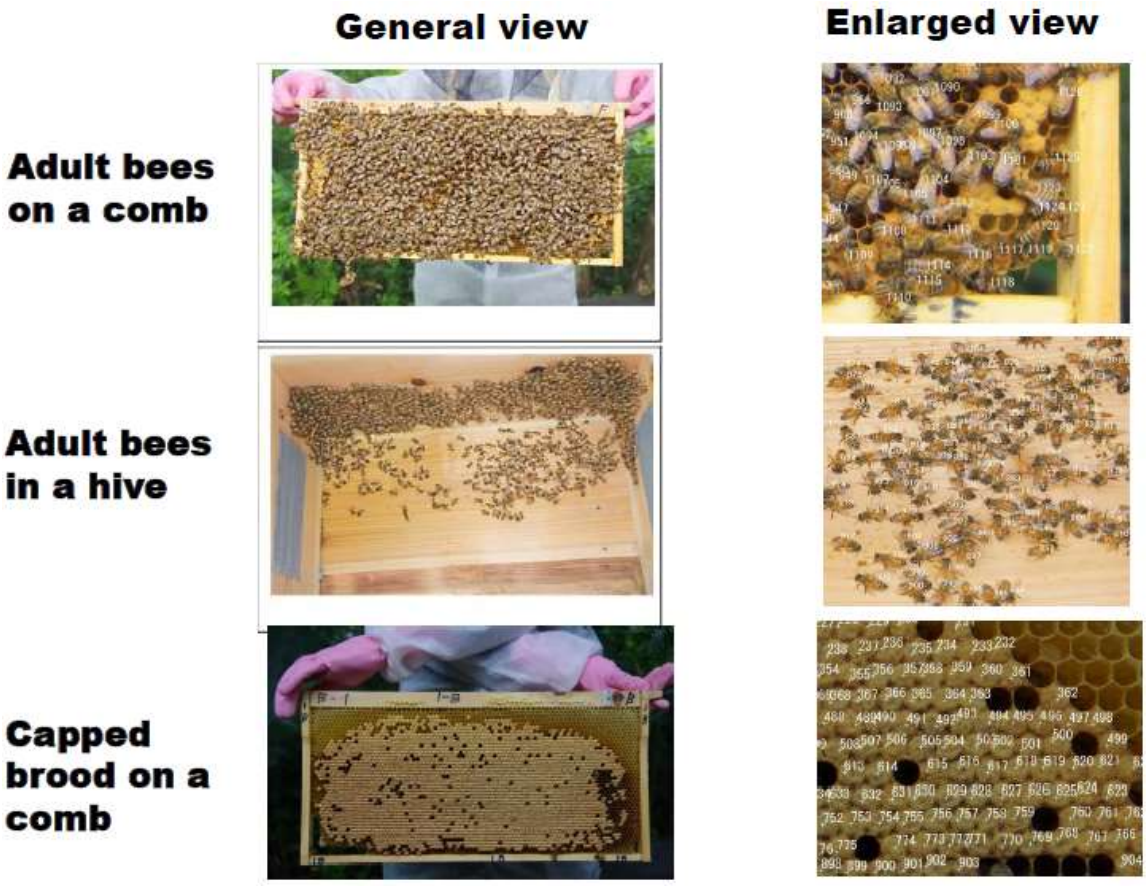


**Figure 1:** Counting method of adult bees and capped brood in a hive. We visually counted almost all of adult bees and capped brood in the hive with numbering bees and capped brood on each photo taken early in the morning before foraging bees went out with the aid of the automatic counting software system, Nanosystem Corporation, Japan.

in an experimental colony of this work did not take only toxic sugar syrup in a beehive but also nectar which is controlled in order to be non-toxic by organically-grown flowers in our apiary, while those in a colony of an actual apiary take nectar which is toxic and/or non-toxic in fields. In addition to that, not only foraging bees but also house bees may take sugar syrup in this work, while only foraging bees take nectar in fields in an actual apiary. Despite these differences from an actual apiary, we believe that this experiment can possibly replicate most of the phenomena occurring in an actual apiary though we have to pay attention to them.

## RESULTS

### Long-term observations

The experiment was conducted under the nearly natural environment where honeybees can freely take foods in fields if they do not like to take toxic sugar syrup in a beehive. We found that the dinotefuran colony (RUN2) became extinct on August 16th but the fenitrothion colony (RUN3) survived long after that day. In the subsequent recovery experiment where pesticide-free sugar syrup and pollen paste were fed to the colony, the fenitrothion colony continued to survive after it succeeded in overwintering. Details of observations are as follows:

In the acclimatization period from June 28th to July 21st in 2012, different numbers of adult bees and capped brood among colonies on June 28th became almost the same on July 21st when the pesticide-administration experiment started just after we had taken photographs of every comb on which honeybees existed in each beehive, those of the remaining honeybees in each beehive after every comb with honeybees was removed and those of every comb which honeybees were shaken off and any honeybees did not exist.

We administered each pesticide (dinotefuran and fenitrothion) into the colony on July 21st and continued till August 16th when the dinotefuran colony (RUN2) became extinct but the fenitrothion colony (RUN3) survived. In the administration period of pesticide, fresh sugar syrup with each pesticide newly prepared was fed into each colony four times on July 21st, 27th, August 3rd and 8th. In order to assess

the acutely toxic impact of each pesticide on a honeybee colony, we conducted observational experiments on the day after the administration date when fresh toxic sugar syrup was administered (July 22nd, 28th and August 4th). We discontinued the administration of fenitrothion and began to feed pesticide-free sugar syrup into the fenitrothion colony (RUN3) on August 16th similar to the control colonies (RUN1 and RUN4). The colony in which dinotefuran was administered (RUN2) rapidly dwindled away to nothing within a month from the start of pesticide administration, but the colony where fenitrothion was administered (RUN3) and both control ones (RUN1 and RUN4)) succeeded in overwintering without extinction. We judged that both control colonies and fenitrothion succeeded in overwintering on February 1st in 2013. We administered a preventive medicine for foul brood following the instructions of Japan Beekeeping Association on March 17th 2013. We finished the experiment on May 10th 2013 after good results of the foul brood test conducted by the Livestock Health Center in Ishikawa Prefecture in Japan because the colonies became very vigorous. After that we continued to observe these vigorous three colonies (RUN1, 3 and 4) till July 14th 2013 for the investigation of the year round behavior of honeybee colony. The queen existed in every colony till the colony became extinct.

All the dinotefuran colonies where the neonicotinoid dinotefuran was administered ended in extinction during the long-term field experiments of our previous work (Yamada et al., 2012) and this work which were conducted from July, 2010 to May, 2013 with different courses depending on their concentration and administration period. On the other hand, the fenitrothion colony dwindled during the administration of fenitrothion assuming a similar aspect to acute toxicity but it rapidly recuperated the vigor after the discontinuance of the administration as earlier described in this work. As a consequence, the fenitrothion colony succeeded in overwintering similar to the control colony. It is desirable that our findings on the fenitrothion colony having been obtained from only one colony in this work should be replicated by other experiments.

**Measurement of number of dead bees**

The number of dead bees in an interval between two adjacent observation dates existing inside (on the bottom and in a feeder) and outside (mainly the front) of the beehive was measured. Table 3 shows the number of dead bees in an interval at every observation date. These results were illustrated in Figure 2 after the conversion of the number of dead bees in an interval between two adjacent observations into the number of dead bees per day (daily number of dead bees). The followings can be seen from Table 3 and Figure 2:

In experimental colonies (RUN2 with dinotefuran and RUN3 with fenitrothion) many dead bees occurred just after the first administration date from July 21st to 22nd. In RUN2 with dinotefuran more than half (52.7%) of initial adult bees were instantly killed and in RUN3 with fenitrothion about one tenth (9.7%) of adult bees died instantly. Dead bees tended to occur for a period of only one day just after the administration date of a newly-prepared pesticide, from July 21st to 22nd (4838 in the dinotefuran colony (DF), 865 in the fenitrothion colony (FT) from 27th to 28th (284 in FT and 314 in FT) and from August 3rd to 4th (81 in DF and 307 in FT), much more than for the subsequent period of several days, from July 22nd to 27th (2682 in DF and 216 in FT), from July 28th to August 3rd (276 in DF, 166 in FT) and August 4thto 8th (318 in DF, 132 in FT), respectively. Average dead bees per day are 4, 838 in DF and 865 in FT from July 21st to 22nd, 536.4 in DF and 43.2 in FT from July 22nd to 27th, 284 in DF and 314 in FT from July 27th to 28th, 46 in DF and 27.7 in DF, 81 in DF and 307 in FT from August 3rd to 4th, and 79.5 in DF and 33 in FT from August 4th to 8th. Such a tendency was more strongly in evidence for the fenitrothion colony (RUN3) than for the dinotefuran colony (RUN2). These results suggest that the organophosphate fenitrothion has a much lower chronic toxicity than the neonicotinoid dinotefuran. In control colonies (RUN1 and RUN4), any dead bees hardly occurred except in cases of attack by Asian giant hornets and death in overwintering.

**Measurement of number of adult bees and capped brood**

Table 4 shows the numbers of adult bees and capped brood in this work. In this table, figures written in red denote values in administration periods of pesticides and black ones denote in pesticide-free periods. Figures 4 and 5 show the changes in the numbers of adult bees and capped brood, respectively. We can find that dinotefuran can affect adult bees much more adversely than fenitrothion which has the same insecticidal activity for stinkbugs as dinotefuran while both of the pesticides can affect brood adversely to about the same degree.

The dinotefuran colony (RUN2) shows a drastic decrease of 46.7% in the number of adult bees within a day from July 21st to July 22nd in comparison with the initial number on July 21st. The decrease in the number of adult bees (4288; 46.7%) is lesser than the number of dead bees (4838; 52.7%) in the same interval. This suggests that almost all of dead bees died on the spot considering the number of newborn adult bees within a day. The dinotefuran colony became rapidly extinct within a month on August 16th when none of the adult bees and capped brood existed.

The fenitrothion colony (RUN3) shows a decrease of 13.3% in the number of adult bees within a day from July 21st to 22nd in comparison with the initial number on July 21st. The decrease in the number of adult bees (1193; 13.3%) is somewhat more than the number of dead bees (865; 9.7%) in the same interval. This suggests that most of the dead bees died on the spot and some of them got lost. The fenitrothion

**Table 3:** Number of dead bees in each interval.

| Date | RUN1 | RUN2 | RUN3 | RUN4 | Note |
|---|---|---|---|---|---|
| | Control 1 | Dinotefuran | Fenitorothion | Control 2 | |
| | Without pesticide | 2 ppm | 10 ppm | Without pesticide | |
| 8-Jul-12 | 2 | 10 | 2 | 1 | |
| 15-Jul-12 | 18 | 1 | 0 | 0 | |
| 21-Jul-12 | 2 | 5 | 3 | 3 | Beginning of pesticide administration |
| 22-Jul-12 | 0 | 4838 | 865 | 0 | Instant death |
| 27-Jul-12 | 10 | 2682 | 216 | 3 | |
| 28-Jul-12 | 0 | 284 | 314 | 0 | Instant death |
| 3-Aug-12 | 4 | 276 | 166 | 7 | |
| 4-Aug-12 | 9 | 81 | 307 | 2 | Instant death |
| 8-Aug-12 | 2 | 318 | 132 | 1 | |
| 16-Aug-12 | 6 | 23 | 120 | 6 | |
| 25-Aug-12 | 0 | | 16 | 1 | |
| 6-Sep-12 | 2 | | 1 | 32 | |
| 15-Sep-12 | 24 | | 34 | 7 | |
| 21-Sep-12 | 0 | | 2 | 389 | Attacks by Asian giant hornets |
| 5-Oct-12 | 14 | | 6 | 1017 | Attacks by Asian giant hornets |
| 19-Oct-12 | 51 | | 5 | 9 | |
| 25-Nov-12 | 56 | | 23 | 215 | |
| 13-Dec-12 | 122 | | 42 | 648 | Attacks by Asian giant hornets |
| 1-Feb-13 | 185 | | 115 | 317 | Natural death in winter |
| 1-Mar-13 | 34 | | 21 | 13 | |
| 9-Mar-13 | 3 | | 0 | 2 | |
| 17-Mar-13 | 10 | | 3 | 5 | |
| 23-Mar-13 | 6 | | 11 | 15 | |
| 29-Mar-13 | 16 | | 16 | 20 | |
| 6-Apr-13 | 29 | | 37 | 32 | |
| 13-Apr-13 | 33 | | 19 | 20 | |
| 19-Apr-13 | 11 | | 24 | 22 | |
| 26-Apr-13 | 32 | | 8 | 66 | |
| 3-May-13 | 57 | | 240 | 99 | Mainly drones |
| 10-May-13 | 99 | | 143 | 100 | Attacks by Asian giant hornets |

colony (RUN3) shows a decrease of 33.3% in the number of adult bees and a decrease of 93.0% in the number of capped brood on August 16th in comparison with the initial number on July 21st.

At the elapse of 26 days the decrement of adult bees is 352.81 bees/day (9173 bees/26 days) in the dinotefuran colony and 114.69 bees/day ((8943-5961) bees/26 days) in the fenitrothion colony. It can be seen from this that dinotefuran resulted in a decrease in the number of the adult bees in the colony about thrice faster than that of fenitrothion.

According to the recovery experiment of the fenitrothion colony from August 16th when the dinotefuran colony became extinct, it was found that the fenitrothion colony began to recover from the adverse effects of fenitrothion immediately after the discontinuance of its administration. The number of capped brood reached the minimum (7% of the initial) at the stop of fenitrothion administration on August 16th and it immediately began to increase. The number of adult bees in the fenitrothion colony reached the minimum (60% of the initial) on September 6th after 21 days elapsed from August 16th when pesticide-free sugar syrup was fed into the fenitrothion colony. These facts suggest that fenitrothion adversely affects the oviposition of the queen during administration of fenitrothion but the adverse effect becomes virtually absent in a short period of time. The delay of 21 days to the minimum number of adult bees from that of capped brood seems to be due to the period for capped brood group of minimum number to grow into the adult bee group of minimum number. The fenitrothion colony increased in the numbers of adult bees and capped brood rapidly as both control colonies after overwintering.

On the other hand, every neonicotinoid colony such as the dinotefuran colony kept alive during the administration of a pesticide became extinct even after the discontinuance of the pesticide administration as reported by Yamada et al. (2012, 2018) because the pesticide stored in the beehive and/or ingested by honeybees most probably continued to affect chronically and adversely over a prolonged period of time. This indicates that organophosphates such as fenitrothion can hardly exert a long-term effect on a honeybee colony and the chronic toxicity can be neglected. Though the control colony of RUN4 was attacked by Asian giant hornets with some bees being killed and it is not very much affected by

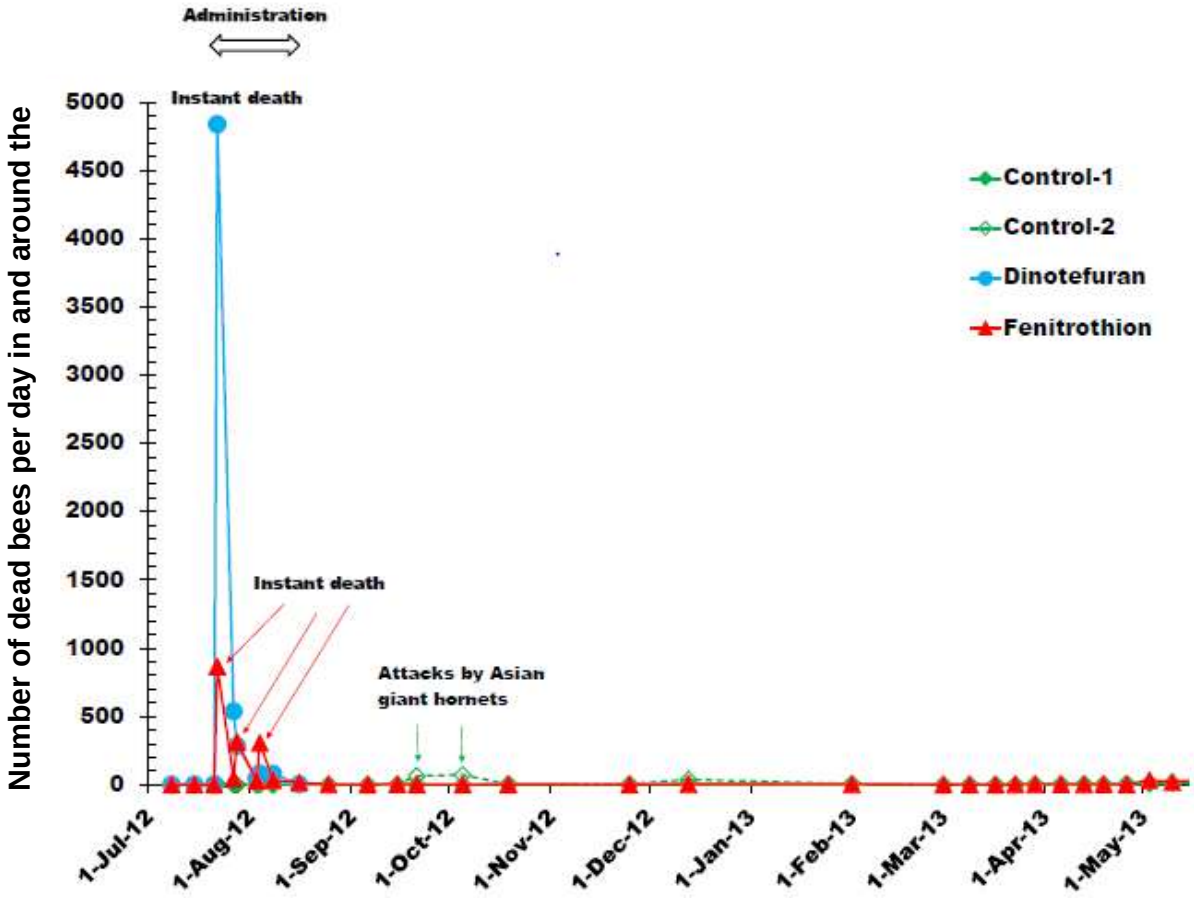


**Figure 2:** Daily number of dead bee. "Control 1, 2", "Dinotefuran" and "Fenitrothion" indicate the colonies supplied with sugar syrup containing no pesticide, dinotefuran and fenitrothion, respectively. These pesticides were administered into their target colonies from July 21st to August 16th 2012. We defined a death of honeybees within a day after the administration of the pesticide (dinotefuran) as an instant death. The massive death of honeybees in Control-2 between September 21st and October 5th were supposed to be caused by the attacks of Asian giant hornets because we found dead and alive Asian giant hornets in front of the hive.

them.

**The number of newly emerging adult bees estimated from capped brood in an interval between two adjacent observation dates**

We may logically assume that the number of honeybees which are contaminated by the pesticide during its administration period probably can be expressed by the sum of the initial number of adult bees at the start of the experiment, the number of adult bees which have newly emerged from capped brood during the pesticide-administration period and the number of brood at the final administration date or at the colony extinction. At the moment, we estimated the number of adult bees which are newly-emerging from capped brood (pupae) in an interval between two adjacent observation dates under the following assumptions: (1) The age distribution of the capped brood at an observation date is uniform between the first day when the cells of larvae are newly capped and the twelfth day when they enclose. (2) The number of adult bees that emerge from the pupae (capped brood) per day at a given day is one-twelfth of the number of the capped brood at the last observation date before the day. (3) The number of adult bees born in an interval between two successive observation dates is given by the product of one-twelfth of the number of the capped brood at the former observation date and the number of days from the former to the latter. (4) The procedure in the assumption (3) is applied even when the number of days between the two successive observation dates is greater than 12. (5) The number of capped brood on the final pesticide-administration date (or final feeding date in the control colony) when a colony stays alive before wintering (fenitrothion colony in this study) or the extinction date when a colony has already become extinct is regarded as the number of adult bees having ingested the pesticide assuming that all the capped brood has already

**Table 4:** Numbers of adult bees and capped brood.

| Date | Elapsed days | Days from pesticide administration | RUN1 (Control 1) Without pesticide | | RUN2 (Starcklemate) Dinotefuran: 2 ppm | | RUN3 (Sumithion) Fenitrothion: 10 ppm | | RUN4 (Control 2) Without pesticide | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | Adult bee | Capped brood | Adult bee | Capped brood | Adult bee | Capped brood | Adult bee | Capped brood |
| 6/28/2012 | 0 | -23 | 7136 | 4746 | 7119 | 5679 | 5690 | 4012 | 5832 | 5094 |
| 7/8/2012 | 10 | -13 | 9621 | 5806 | 8877 | 6446 | 7157 | 5286 | 8917 | 4710 |
| 7/15/2012 | 17 | -6 | 8695 | 10215 | 6878 | 13267 | 7565 | 9619 | 8265 | 11143 |
| 7/21/2012 | 23 | 0 | 9647 | 10254 | 9173 | 9442 | 8943 | 8732 | 9665 | 11301 |
| 7/22/2012 | 24 | 1 | 10136 | 10210 | 4885 | 8834 | 7750 | 8694 | 9558 | 10967 |
| 7/27/2012 | 29 | 6 | 10633 | 10617 | 1434 | 4548 | 8721 | 6563 | 10770 | 10329 |
| 7/28/2012 | 30 | 7 | 10391 | 10858 | 1313 | 3891 | 7786 | 6389 | 10901 | 10581 |
| 8/3/2012 | 36 | 13 | 12083 | 10000 | 1049 | 1131 | 8289 | 3390 | 11939 | 10025 |
| 8/4/2012 | 37 | 14 | 12389 | 9687 | 771 | 840 | 7559 | 2901 | 12041 | 10269 |
| 8/8/2012 | 41 | 18 | 14065 | 7154 | 33 | 208 | 6625 | 1352 | 12978 | 8472 |
| 8/16/2012 | 49 | 26 | 13371 | 6111 | 0 | 0 | 5961 | 607 | 12207 | 5977 |
| 8/25/2012 | 58 | 35 | 11961 | 6014 | | | 4467 | 918 | 10997 | 6684 |
| 9/6/2012 | 70 | 47 | 11165 | 8783 | | | 3534 | 3406 | 11582 | 8126 |
| 9/15/2012 | 79 | 56 | 11980 | 5531 | | | 4576 | 4187 | 11825 | 7135 |
| 9/21/2012 | 85 | 62 | 12166 | 6086 | | | 5859 | 4119 | 11025 | 9202 |
| 10/5/2012 | 99 | 76 | 10715 | 7615 | | | 6593 | 4648 | 10510 | 5679 |
| 10/19/2012 | 113 | 90 | 11726 | 7280 | | | 7326 | 4713 | 10038 | 6628 |
| 11/25/2012 | 150 | 127 | 13255 | 36 | | | 7755 | 10 | 12477 | 2937 |
| 12/13/2012 | 168 | 145 | 12858 | 0 | | | 7080 | 0 | 13316 | 305 |
| 2/1/2013 | 218 | 195 | 9306 | 0 | | | 5652 | 0 | 9421 | 0 |
| 3/1/2013 | 246 | 223 | 7464 | 17 | | | 5957 | 26 | 8426 | 0 |
| 3/9/2013 | 254 | 231 | 7512 | 497 | | | 6358 | 619 | 8017 | 660 |
| 3/17/2013 | 262 | 239 | 6862 | 1691 | | | 6152 | 2329 | 7372 | 2419 |
| 3/23/2013 | 268 | 245 | 7312 | 2431 | | | 6470 | 3217 | 7416 | 3624 |
| 3/29/2013 | 274 | 251 | 7720 | 4097 | | | 7143 | 4994 | 8018 | 5165 |
| 4/7/2013 | 283 | 260 | 9518 | 7326 | | | 8797 | 7053 | 9833 | 8414 |
| 4/13/2013 | 289 | 266 | 12523 | 10166 | | | 12038 | 8670 | 12594 | 11211 |
| 4/19/2013 | 295 | 272 | 15677 | 11324 | | | 14275 | 10320 | 16221 | 12975 |
| 4/26/2013 | 302 | 279 | 20574 | 9725 | | | 17132 | 10803 | 20412 | 14287 |
| 5/3/2013 | 309 | 286 | 23935 | 5808 | | | 20413 | 9631 | 24100 | 14521 |
| 5/10/2013 | 316 | 293 | 23629 | 4551 | | | 16477 | 9020 | 27670 | 13380 |

**Note:** Red numbers shows an administration period of pesticide.

ingested the pesticide. Exceptionally, when the number of the capped brood at the colony extinction is zero, the number of newly-emerging adult bees during the final interval is assumed to be equal to the number of the capped brood at the last observation before the colony extinction or the final pesticide-administration in the case where the colony become extinct after the pesticide discontinues to be administered.

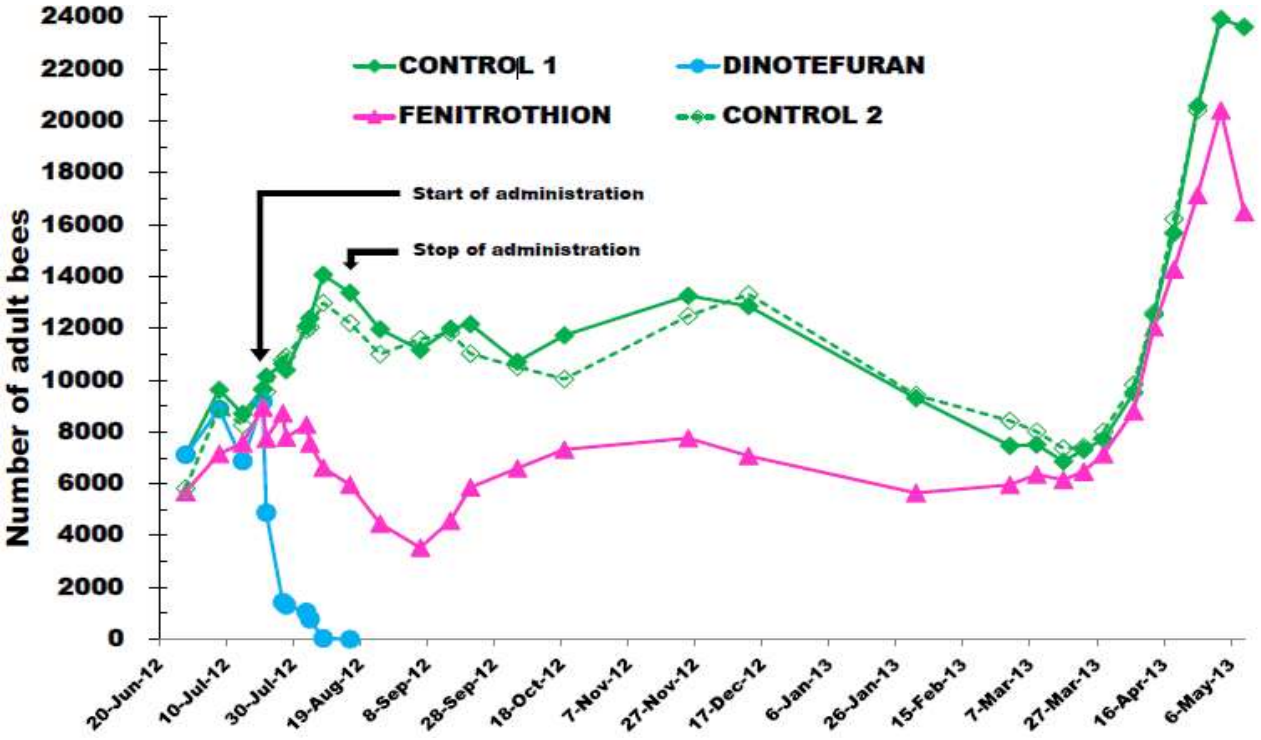


**Figure 3:** Change in the number of adult bees. "Control 1, 2", "dinotefuran" and "fenitrothion" indicate the colonies supplied with sugar syrup containing no pesticide, dinotefuran and fenitrothion, respectively. These pesticides were administered into their target colonies from July 21st to August 16 2012. The queen existed in every colony till the end of each experiment; that is, the queen in the dinotefuran colony existed till extinction.

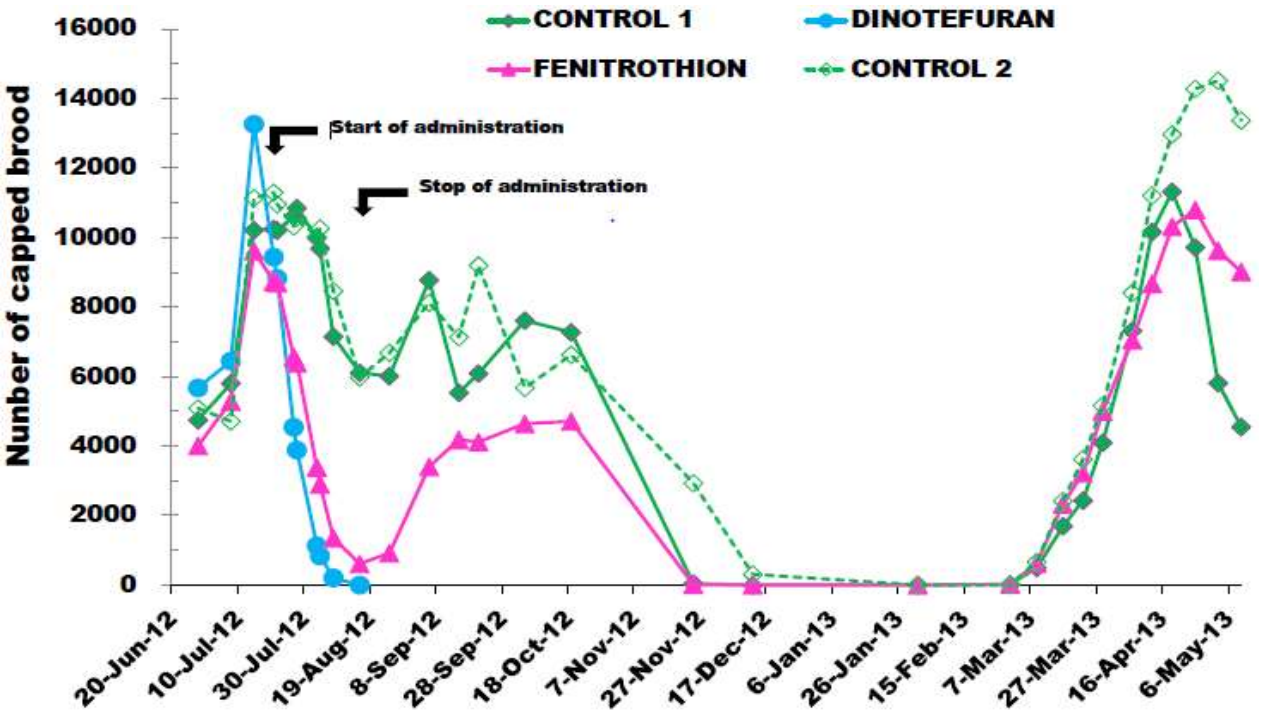


**Figure 4:** Change in the number of capped brood. "Control 1, 2", "dinotefuran" and "fenitrothion" indicate the colonies supplied with sugar syrup containing no pesticide, dinotefuran and fenitrothion, respectively. These pesticides were administered into their target colonies from July 21st to August 16th 2012.

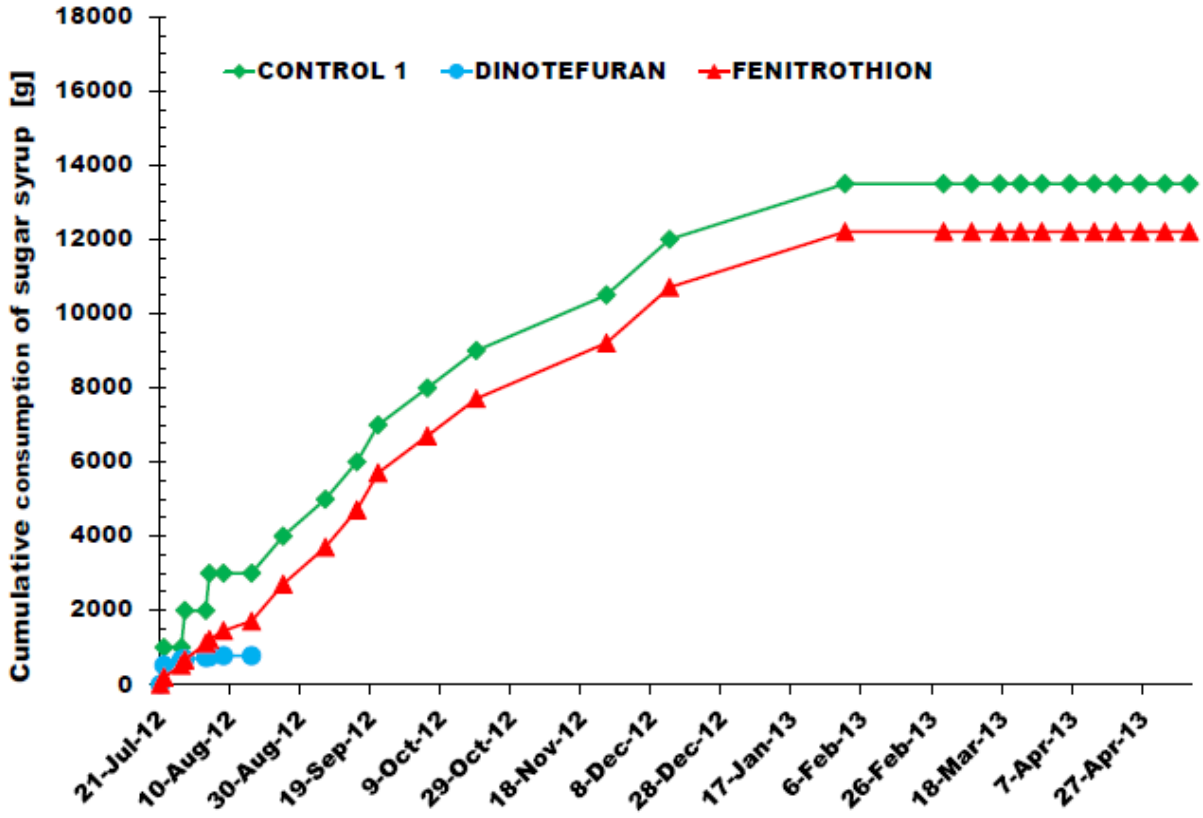


**Figure 5:** Cumulative consumption of sugar syrup by each colony. "Control 1", "dinotefuran" and "fenitrothion" indicate the colonies supplied with sugar syrup containing no pesticide, dinotefuran and fenitrothion, respectively. These pesticides were administered into their target colonies from July 21st to August 16th 2012. Control 2 shows the same curve as Control 1.

**The total number of honeybees during the administration period of pesticide**

As the total number of honeybees during the administration period of pesticide is given by finding the sum of the number of initial adult bees which have already existed at the start of experiment, the number of newly emerging adult bees during the administration period and the number of the capped brood at the end of the administration on the assumption that the capped brood have probably taken the pesticide.

Here, we concretely explained the procedure to obtain the total number of honeybees from the start of experiment to the discontinuance of experiment or to the colony extinction using two examples of the dinotefuran colony (RUN2) and the fenitrothion colony (RUN3). We administered a pesticide (dinotefuran and fenitrothion) into a colony on July 21st 2012 and discontinued to administer it on August 16th when the dinotefuran colony (RUN2) became extinct, though the fenitrothion colony survived. For the dinotefuran colony (RUN2), the number of initial adult bees is 9173; the number of newly-emerging adult bees from capped brood (pupae) in an interval between two successive observation dates = (9442/12)(1) from July 21st to 22nd + (8834/12)(5) from July 22nd to 27th + (4548/12)(1) from July 27th to 28th + (3891/12)(6) from July 28th to August 3rd + (1131/12)(1) from August 3rd to 4th + (840/12)(4) from August 4th to 8th = 7166.4, and the number of newly-emerging adult bees during the final interval from August 8th to 16th, where they seem to have taken the pesticide (dinotefuran) before being capped is 208, that is, the number of capped brood on August 8th, because capped brood was zero at the colony extinction on August 16th. That is, the total number of honeybees which have taken the pesticide (dinotefuran) in the dinotefuran colony during the administration period is the sum (16547.4) of the number of the initial adult bees (9173), the number of newly-emerging adult bees (7166.4) and the number of the final capped brood (208).

In like manner, for the fenitrothion colony (RUN3), the number of initial adult bees is 8, 943; the number of newly-emerging adult bees between two successive observation dates = (8732/12)(1) from July 21st to 22nd + (8694/12)(5) from July 22nd to 27th + (6563/12)(1) from July 27th to 28th + (6389/12)(6) from July 28th to August 3rd + (3390/12)(1) from August 3rd to 4th + (2901/12)(4) from August 4th to 8th + (1352/12)(8) from August 8th to 16th = 10242.4. The number of the capped brood at the stop of administration of the pesticide (fenitrothion) on August 16th was 607, all of

**Table 5:** Interval and cumulative consumptions of sugar syrup [g].

| Date | Elapsed days | RUN1 (Control 1) Without pesticide | | RUN2 (Dinotefuran) 2 ppm | | RUN3 (Fenitrothion) 10 ppm | | RUN4 (Control 2) Without pesticide | |
|---|---|---|---|---|---|---|---|---|---|
| | | Interval consumption | Cumulative consumption | Interval consumption | Cumulative consumption | Interval consumption | Cumulative consumption | Interval consumption | Cumulative consumption |
| 21-Jul-12 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 22-Jul-12 | 1 | 1000 | 1000 | 518 | 518 | 195 | 195 | 1000 | 1000 |
| 27-Jul-12 | 6 | 0 | 1000 | 145 | 663 | 324 | 519 | 0 | 1000 |
| 28-Jul-12 | 7 | 1000 | 2000 | 15 | 678 | 145 | 664 | 1000 | 2000 |
| 3-Aug-12 | 13 | 0 | 2000 | 50 | 728 | 452 | 1116 | 0 | 2000 |
| 4-Aug-12 | 14 | 1000 | 3000 | 20 | 748 | 100 | 1216 | 1000 | 3000 |
| 8-Aug-12 | 18 | 0 | 3000 | 28 | 776 | 239 | 1455 | 0 | 3000 |
| 16-Aug-12 | 26 | 0 | 3000 | 0 | 776 | 252 | 1707 | 0 | 3000 |
| 25-Aug-12 | 35 | 1000 | 4000 | | | 1000 | 2707 | 1000 | 4000 |
| 6-Sep-12 | 47 | 1000 | 5000 | | | 1000 | 3707 | 1000 | 5000 |
| 15-Sep-12 | 56 | 1000 | 6000 | | | 1000 | 4707 | 1000 | 6000 |
| 21-Sep-12 | 62 | 1000 | 7000 | | | 1000 | 5707 | 1000 | 7000 |
| 5-Oct-12 | 76 | 1000 | 8000 | | | 1000 | 6707 | 1000 | 8000 |
| 19-Oct-12 | 90 | 1000 | 9000 | | | 1000 | 7707 | 1000 | 9000 |
| 25-Nov-12 | 127 | 1500 | 10500 | | | 1500 | 9207 | 1500 | 10500 |
| 13-Dec-12 | 145 | 1500 | 12000 | | | 1500 | 10707 | 1500 | 12000 |
| 1-Feb-13 | 195 | 1500 | 13500 | | | 1500 | 12207 | 1500 | 13500 |
| 1-Mar-13 | 223 | 0 | 13500 | | | 0 | 12207 | 0 | 13500 |
| 9-Mar-13 | 231 | 0 | 13500 | | | 0 | 12207 | 0 | 13500 |
| 17-Mar-13 | 239 | 0 | 13500 | | | 0 | 12207 | 0 | 13500 |
| 23-Mar-13 | 245 | 0 | 13500 | | | 0 | 12207 | 0 | 13500 |
| 29-Mar-13 | 251 | 0 | 13500 | | | 0 | 12207 | 0 | 13500 |
| 6-Apr-13 | 259 | 0 | 13500 | | | 0 | 12207 | 0 | 13500 |
| 13-Apr-13 | 266 | 0 | 13500 | | | 0 | 12207 | 0 | 13500 |
| 19-Apr-13 | 272 | 0 | 13500 | | | 0 | 12207 | 0 | 13500 |
| 26-Apr-13 | 279 | 0 | 13500 | | | 0 | 12207 | 0 | 13500 |
| 3-May-13 | 309 | 0 | 13500 | | | 0 | 12207 | 0 | 13500 |
| 10-May-13 | 316 | 0 | 13500 | | | 0 | 12207 | 0 | 13500 |

**Note:** Red figures denote toxic sugar syrup with the pesticide (dinotefuran or fenitrothion).

which seemed to have taken the pesticide. That is, the total number of honeybees which took the pesticide in the fenitrothion colony (RUN3) is the sum (19792.4) of the number of the initial adult bees (18943), the number of newly-emerging adult bees (10242.4) and the number of the final capped brood (607).

## Intake of pesticide by a colony

Figure 5 shows the cumulative consumption of sugar syrup by each colony, while Table 5 shows the interval consumption of toxic sugar syrup with each pesticide ingested by the dinotefuran colony and the fenitrothion during an interval between two successive observation dates and the cumulative total consumption of sugar syrup from July 21$^{st}$ 2012 to August 16$^{th}$. The cumulative total consumption of sugar syrup by the dinotefuran colony is 776 g and that by the fenitrothion colony is 1707 g during the administration period of a pesticide (dinotefuran or fenitrothion) from July 21$^{st}$ to August 16$^{th}$.

**Table 6:** Interval and daily consumption of toxic sugar syrup from the start of administration (July 21st) to the finish (August 16th).

| Date | Elapsed days | RUN2 (Dinotefuran: 2ppm) | | RUN3 (Fenitrothion : 10 ppm) | | Note |
|---|---|---|---|---|---|---|
| | | Interval consumption of toxic sugar syrup between 2 successive observation dates [g] | Daily consumption of toxic sugar syrup [g/day] | Interval consumption of toxic sugar syrup between 2 successive observation dates [g] | Daily consumption of toxic sugar syrup [g/day] | |
| 21-Jul-12 | 0 | 0 | 0.00 | 0 | 0.00 | Observation date |
| 22-Jul-12 | 1 | 518 | 518.00 | 195 | 195.00 | Observation date |
| 23-Jul-12 | 2 | | 29.00 | | 64.80 | |
| 24-Jul-12 | 3 | | 29.00 | | 64.80 | |
| 25-Jul-12 | 4 | | 29.00 | | 64.80 | |
| 26-Jul-12 | 5 | | 29.00 | | 64.80 | |
| 27-Jul-12 | 6 | 145 | 29.00 | 324 | 64.80 | Observation date |
| 28-Jul-12 | 7 | 15 | 15.00 | 145 | 145.00 | Observation date |
| 29-Jul-12 | 8 | | 8.33 | | 75.33 | |
| 30-Jul-12 | 9 | | 8.33 | | 75.33 | |
| 31-Jul-12 | 10 | | 8.33 | | 75.33 | |
| 1-Aug-12 | 11 | | 8.33 | | 75.33 | |
| 2-Aug-12 | 12 | | 8.33 | | 75.33 | |
| 3-Aug-12 | 13 | 50 | 8.33 | 452 | 75.33 | Observation date |
| 4-Aug-12 | 14 | 20 | 20.00 | 100 | 100.00 | Observation date |
| 5-Aug-12 | 15 | | 7.00 | | 59.75 | |
| 6-Aug-12 | 16 | | 7.00 | | 59.75 | |
| 7-Aug-12 | 17 | | 7.00 | | 59.75 | |
| 8-Aug-12 | 18 | 28 | 7.00 | 239 | 59.75 | Observation date |
| 9-Aug-12 | 19 | | 0.00 | | 31.50 | |
| 10-Aug-12 | 20 | | 0.00 | | 31.50 | |
| 11-Aug-12 | 21 | | 0.00 | | 31.50 | |
| 12-Aug-12 | 22 | | 0.00 | | 31.50 | |
| 13-Aug-12 | 23 | | 0.00 | | 31.50 | |
| 14-Aug-12 | 24 | | 0.00 | | 31.50 | |
| 15-Aug-12 | 25 | | 0.00 | | 31.50 | |
| 16-Aug-12 | 26 | 0 | 0.00 | 252 | 31.50 | Observation date |

**Note:** Blue numbers are estimated from the consumption of sugar syrup measured at an observation date under the assumption that the consumption per day (consumption rate) is same between two successive observation dates from a certain observation date to the previous one: E.g.,, the consumption rate from July 23rd to 27th is obtained from dividing the consumption measured on July 27th (145 g) by the interval (5 days) for RUN 2.

Assuming that the consumption of toxic sugar syrup per day is constant between two successive observation dates, the daily consumption can be estimated (Table 6). It can be seen from Table 6 that the dinotefuran colony ingested about 67% (518 g/776 g) of the cumulative total consumption of toxic sugar syrup only within one day just after the first administration but the fenitrothion colony did not more than about 11% (195 g/1707 g). From another point of view, the initial daily consumption of toxic sugar syrup by the dinotefuran colony just after the first administration is about 2.7 times (518 g/195 g) as much as that by the fenitrothion colony. This difference may perhaps come from malodorous fenitrothion as opposed to odorless dinotefuran.

The intake of a pesticide by each experimental colony is calculated from the cumulative total consumption of sugar syrup. As the concentration of

dinotefuran in sugar syrup is 2 ppm and that of fenitrothion is 10 ppm, the cumulative total intake of dinotefuran becomes 1.552 mg and that of fenitrothion is 17.07 mg. The cumulative total intake is the amount of each pesticide consumed by the colony from the feeder in the beehive before August 16th when the pesticide administration was discontinued. Some of the cumulative total intake was ingested by honeybees, while the rest of that stored as honey and bee bread in cells on combs after honeybees converted toxic sugar syrup into toxic honey and/or toxic bee bread. When toxic sugar syrup is stored as honey and/or bee bread, honeybees are inevitably affected by the pesticide through the conversion process. We cannot know the impact of the pesticide on honeybees when toxic sugar syrup is converted into honey and/or bee bread. We have to recognize that the entire cumulative total intake of the pesticide is not the actual intake of the pesticide ingested by honeybees but is the apparent intake of the pesticide consumed while being ingested by honeybees and/or being stored in cells of combs, but a difference between the apparent and real intakes seems not to matter so much from a practical standpoint of view because the honey and beebread stored in cells of combs in a beehive will probably be ingested by honeybees sooner or later and the stored amount will not make much difference under the same environmental conditions.

At present, we estimated the intake of pesticide per bee during the administration period of pesticide from dividing each cumulative total intake of dinotefuran or fenitrothion by the total number of honeybees during the administration period of pesticide. We can estimate the intake of pesticide per bee till August 16th when the dinotefuran colony became extinct. The intakes of pesticide per bee is 93.8 ng/bee by dividing 1.552 mg by 16547.4 for the dinotefuran colony (RUN2) and 862.5 ng/bee by dividing 17.07 mg by 19792.4 for the fenitrothion colony (RUN3), respectively. Comparing the intake of dinotefuran per bee with the average $LD_{50}$ for acute oral of a honeybee which is 20.9 ng/bee (7.6+23+32)/3), the ratio of the intake to the average $LD_{50}$ is about 4.5. Similarly, the ratio of the intake of fenitrothion per bee to the $LD_{50}$ for acute oral of a honeybee (200 ng/bee) is about 4.3. We perceived that the intakes of the pesticides per bee are about 4.5 times higher than their $LD_{50}$. This reason seems to be due to the amount of sugar syrup stored in cells on combs, which will probably depend on the weather conditions and the blooming season of flowers etc.

## DISCUSSION

### Why do dinotefuran kill more adult bees than fenitrothion?

Figure 3 shows that adult bees in the dinotefuran colony steeply decreased in number just after the administration of dinotefuran and became extinct in a short period of time. On the other hand, adult bees in the fenitrothion colony gradually decreased in number to about two-thirds of the initial at the discontinuation of fenitrothion administration (at the extinction of the dinotefuran colony). They continued to decrease in number for a while even after the discontinuation of fenitrothion administration. After they reached the minimum (three-fifths of the initial), they began to increase in number during the feeding of pesticide-free sugar syrup (during the recovery experiment) as was the case with the control colonies. On the other hand, we can find from Figure 4 that capped brood in both experimental colonies steeply decreased in number after the administration of the pesticides and reached the minimum (0% for the dinotefuran colony of the initial; 7% for the fenitrothion colony) at the extinction of the dinotefuran colony (at the stop of pesticide administration). Thereafter, the capped brood in fenitrothion colony began to increase in number during the recovery experiment assuming almost the same aspect as the control colonies.

It can be suggested from the aforementioned facts that the insecticidal activity for a honeybee of fenitrothion will probably be much weaker than that of dinotefuran despite their same insecticidal activity for a stinkbug (Figure 9) which shows the daily number of dead bees per adult bee (namely, mortality per day) expressed in value relative to that on July 21st. We can probably understand that the queen was severely adversely affected by the pesticides and her oviposition capacity was reduced when toxic sugar syrup with pesticide was given to the queen as toxic honey or toxic bee bread, and/or the brood were also adversely affected by the pesticides before being capped when toxic honey and toxic bee bread were given to them by house bees. Especially, bee bread seems to be given before the pesticides have lost their toxicity as a result of short period of storage in their cells (Gillian, 1979; DeGrandi-Hoffman et al., 2013).

Currently, we deduced a factor which resulted in the difference between dinotefuran and fenitrothion from the following hypothesis about neurotransmission system. Supposing that the frequency and quantity of acetylcholine (ACh) differ among a brood (larva), an adult bee (worker) and a queen, those of the enzyme acetylcholinesterase (AChE) which generates in order to readily decompose ACh may also differ among them (Dewhurst et al., 1970; Grzelak et al., 1970; Mohamad, 1982; van der Kloot, 1955). That is to say, an adult bee without peculiar behavior produces less ACh and AChE than a brood with feeding behavior and a queen with ovipositional behavior. Assuming that ACh which can activate non-specific cation conductance to directly excite neurons is produced more in a brood which has to aggressively inform a nurse bee that she needs her feed than an adult bee and AChE in the brood becomes more than that in the adult bee. The neonicotinoid dinotefuran acts as an agonist of the ACh receptor by binding to the postsynaptic nicotinic acetylcholine receptor and the nerve is continually stimulated by dinotefuran itself while AChE is not affected by it and dinotefuran act on the nervous system independently of the frequency and quantity of actual ACh.

As a result, dinotefuran seems to exhibit similar toxicity for an adult bee to that for a brood.

On the other hand, as the organophosphate fenitrothion acts on the nervous system as inhibitor of AChE and continued transmission of ACh, fenitrothion strongly affects AChE. As a result, fenitrothion which can decompose AChE probably continue to stimulate the nervous system of a brood stronger than that of an adult bee though dinotefuran which is an acetylcholine mimic and cannot be influenced by AChE which continues to strongly stimulate the nervous system of a brood similar to that of an adult bee regardless of the frequency and quantity of AChE.

Presently, we considered the influence of these pesticides on the nervous system of a queen where ACh seems to generate when she oviposits. Considering that AChE, which generates as ACh generates, is decomposed by fenitrothion; ACh can continue to affect the nervous system of the queen similar to a brood under the condition of little AChE and can reduce her oviposition activity. Dinotefuran mimicking ACh also affect the nervous system of the queen continuously, unaffected by AChE and reduce her oviposition activity, as is the case with fenitrothion. That is, a queen exposed to fenitrothion seems to lay almost the same small number of eggs as dinotefuran.

**Why does the dinotefuran colony consume toxic sugar syrup at the first administration more than the fenitrothion colony?**

Figure 10 shows the consumption of toxic sugar syrup with 2 ppm dinotefuran taken by the colony during each interval between two adjacent observation dates and that of toxic sugar syrup with 10 ppm fenitrothion in this work, respectively. It can be seen from Figure 10 that the dinotefuran colony takes an extremely large quantity of toxic sugar syrup (about 2.7 times) than the fenitrothion colony just after the first administration, when the numbers of adult bees and capped brood in each colony were on almost the same level after the acclimatization period. This tendency can be seen in the daily consumption of toxic sugar syrup per adult bee in Figure 9 which shows the daily consumption of toxic sugar syrup per adult bee expressed in value relative to that on July 21st. These suggest that fenitrothion seems to be more repellent than dinotefuran judging from the facts that organophosphates such as fenitrothion are slightly repellent insecticides as reported by Kegley et al. (2014) but neonicotinoids such as dinotefuran are non-repellent insecticides as reported by Gels et al. (2002), Larson et al. (2013) and BASF (2014).

**Why does fresh toxic sugar syrup with fenitrothion kill more adult bees than older toxic sugar syrup?**

Figure 2 shows that daily dead bees in the fenitrothion colony rapidly increase in number just after feeding newly-prepared toxic sugar syrup with fenitrothion into the beehive and afterwards begin to decrease in number in every administration. On the other hand, the tendency is not clearly visible for those in the dinotefuran colony. The daily number of dead bees is obtained by dividing the number of dead bees in an interval by the number of days in the interval as shown in Table 3. As the number of dead bees in each interval depends on the population to which they belong, we try to obtain the daily number of dead bees per adult bee which is obtained from dividing the daily number of dead bees by the population (Table 4) at the last observation before counting the dead bees which seem to have belonged there.

Figure 9 shows the relative daily number of dead bees per adult bee after the conversion to a logarithmic scale, which is shown in value relative to that on July 21st just before the administration of the pesticide into each experimental colony (0.000103836 heads/day/adult bee on July 21st for the dinotefuran colony and 0.0000605767 heads/day/adult bee for the fenitrothion colony). From Figure 9, we can find that the daily number of dead bees per adult bee for the fenitrothion colony shows the extremely clear tendency in rapid increase and that for the dinotefuran colony shows the slightly visible tendency. Noticeably, the daily number of dead bees per adult bee for the fenitrothion colony is much smaller than that for the dinotefuran colony.

Here, we examined the daily consumption of sugar syrup per adult bee. As the consumption of toxic sugar syrup by honeybees also depends on the population to which they belong, we try to obtain the daily consumption of toxic sugar syrup per adult bee by dividing the daily consumption of toxic sugar syrup (Table 6) by the population (the number of adult bees shown in Table 4) at the last observation before counting the dead bees which seem to have belonged there. Figure 9 shows the daily consumption of toxic sugar syrup per adult bee after the conversion to a logarithmic scale, which is shown in value relative to the average of those by two control colonies for a day between July 21st to 22nd (0.1026 g/day/adult bee; namely, the average of 0.1011 g/day/adult bee in Contro1 1 and 0.1040 g/day/adult bee in Contro1 2.

From Figure 9 we can find that the daily consumption of toxic sugar syrup per adult bee for each experimental colony changes with time. At the elapse of a day after the first administration on July 21st in 2012, the daily consumption of toxic sugar syrup per adult bee by the dinotefuran colony is comparatively greater than that for the fenitrothion colony on July 22nd. After that, a difference in daily consumption per adult bee between dinotefuran colony and fenitothion colony became small. The daily consumption of toxic sugar syrup per adult bee in the fenitothion colony tends to decrease with time. The tendency may be due to a repellent effect of fenitrothion against honeybees.

Examining Figure 9 in details, we can find that the daily number of dead bees per adult bee decreased rapidly from

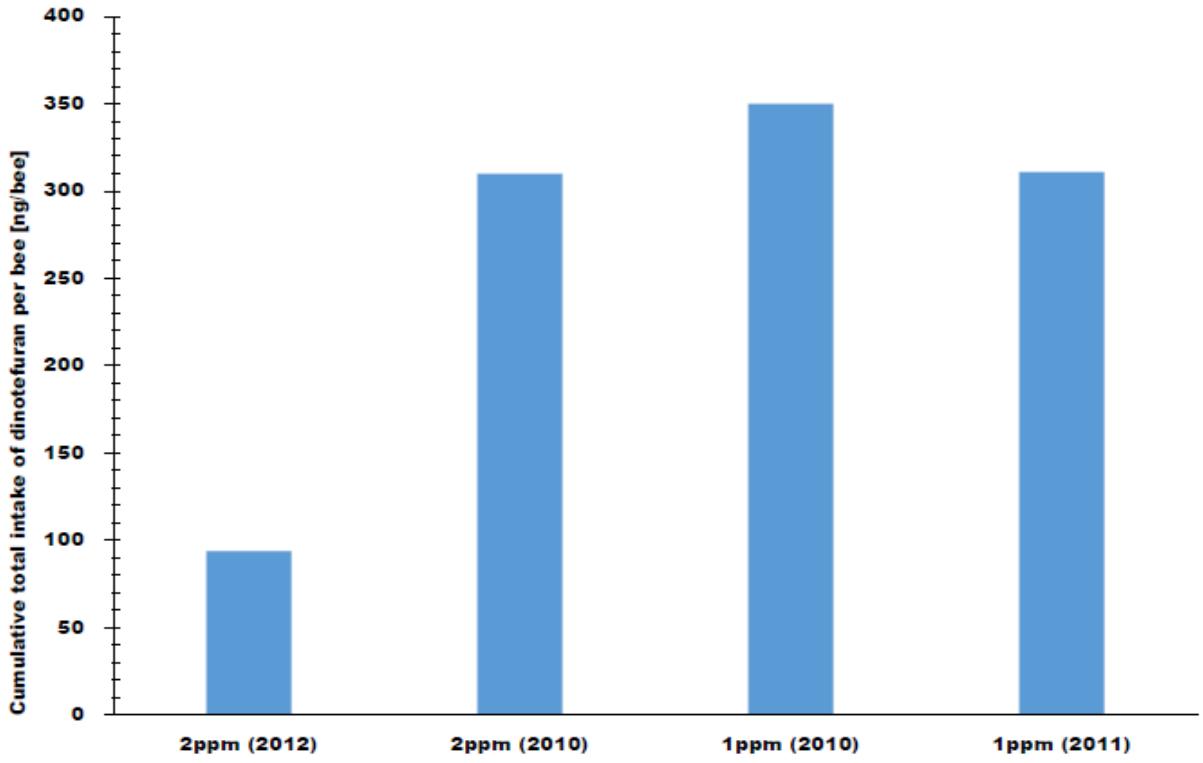


**Figure 6:** Estimated cumulative intake of dinotefuran per bee till the colony extinction in this work and previous ones. We compare the estimated amount of dinotefuran that a honeybee takes till the colony extinction among three kinds of our field experiments which started at 2010, 2011 and 2012. Each concentration such as 2 ppm indicates the concentration of dinotefuran in sugar syrup fed to a colony. The number in the parenthesis indicates the year for each of our field experiments: 2012 indicate this work, while 2010 and 2011 indicate our previous works which have been already reported by Yamada et al. (2012, 2018).

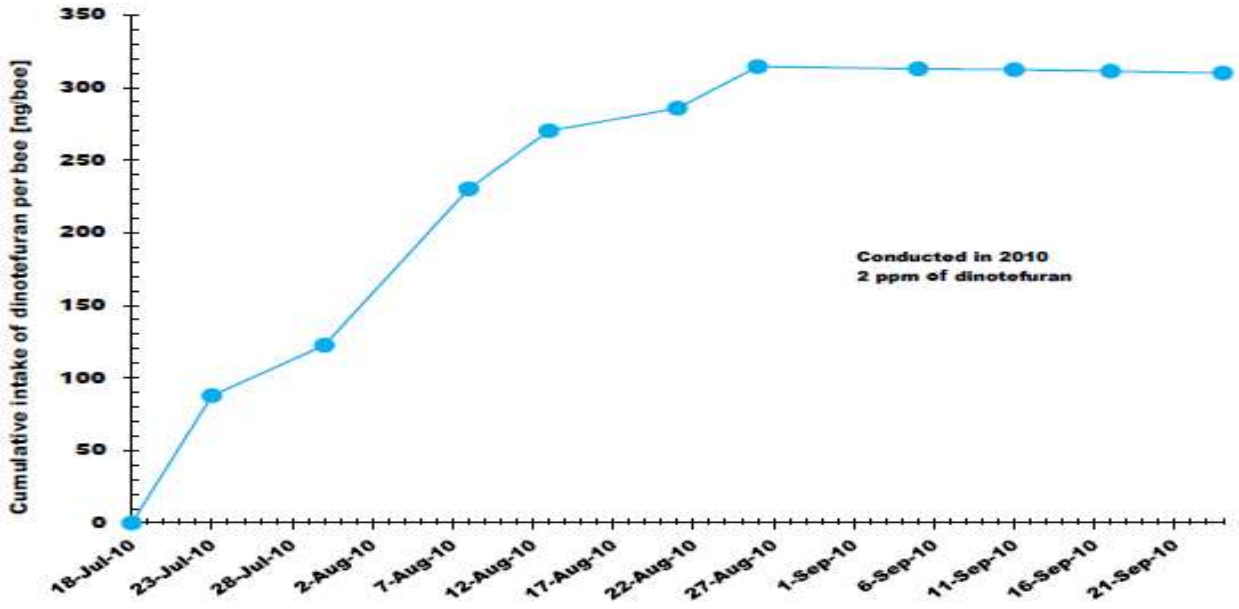


**Figure 7:** Cumulative intake of dinotefuran per bee in 2010. The cumulative intake of dinotefuran can be obtained by dividing a total of the intake by that of honeybees from the start of administration of dinotefuran till a certain observation date when the experiment was conducted in 2010 (Yamada et al., 2012).

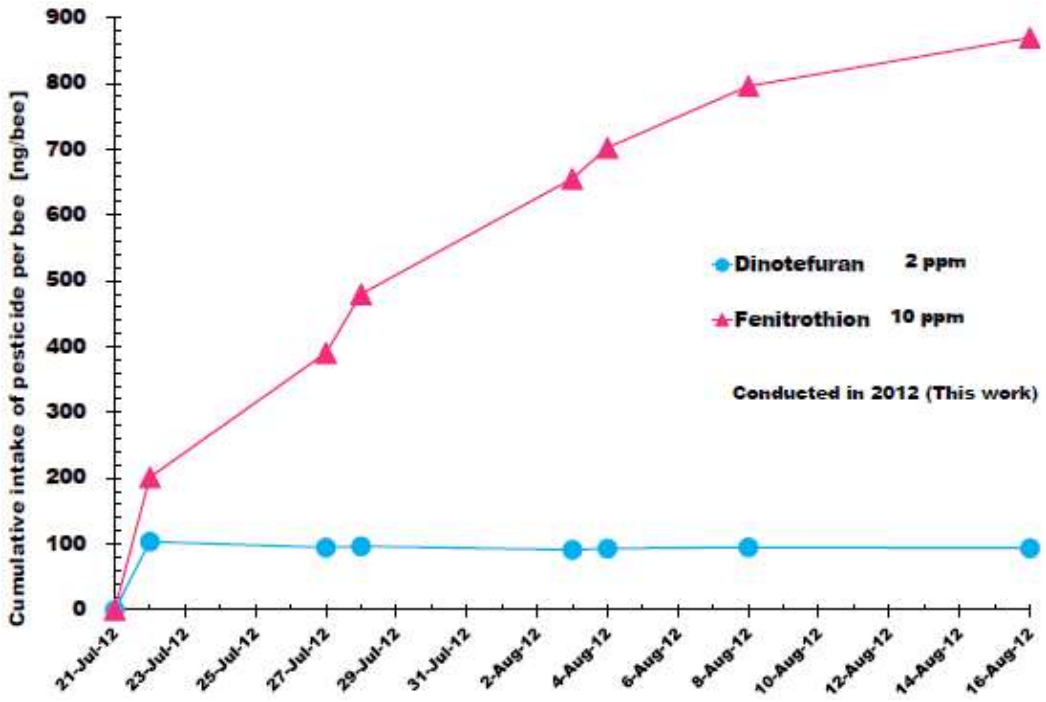


**Figure 8:** Cumulative intakes of dinotefuran and fenitrothion per bee in this work. These cumulative intakes can be obtained by similar procedure to Figure 7.

the day (July 22nd) after the first administration of fenitrothion than dinotefuran, and subsequently it turns to a much sharper increase just after the second administration (July 27th). This tendency recurs with attenuating the amplitude of vibration in every administration from the first administration to the fourth. The daily number of dead bees per adult bee in the dinotefuran colony becomes almost constant keeping its peak after the third administration and that in the fenitrothion colony begins to decrease after the final peak (August 4th). The daily number of dead bees per adult bee keeps the level much higher in the dinotefuran colony than in fenitrothion colony and after the third administration the difference between the two experimental colonies widens. These findings suggest that the insecticidal activity of fenitrothion will probably decrease with time much more rapidly than that of dinotefuran. It seems probable that easy decomposability and short-term persistence of fenitrothion (Pehkonen and Zhang, 2002) can cause the decrease in toxicity with time.

Here, we discussed in detail the daily consumption of toxic sugar syrup per adult bee (Figure 9). The daily consumption of pesticide-free (non-toxic) sugar syrup per adult bee by each colony was not measured before the first administration. Assuming that every daily consumption of pesticide-free sugar syrup per adult bee just before the first administration is almost the same as the average of those by the two control colonies between July 21st and 22nd among all colonies, it is roughly 0.1026 g/day/adult bee which is the average of 0.1011 g/day/adult bee obtained by dividing 1000 g of interval sugar syrup consumption from July 21st to 22nd by (9647+10136)/2=9891.5 adult bees on July 21st for Control 1 (RUN1) and 0.1040 g/day/adult bee similarly obtained by dividing 1000 g of interval sugar syrup consumption by (9665+9558)/2=9611.5 adult bees for Control 2 (RUN4) (Tables 4 and 5). Permitting the aforementioned assumption, the daily consumptions of sugar syrup per adult bee for both the dinotefuran colony and the fenitrothion rapidly decreased just after the first administration between July 21st and 22nd.

The aforementioned rapid decrease in the intake of toxic sugar syrup just after every administration seems to be due to the following reasons: Firstly, the rapid decrease can be caused by the repellent efficacy due to volatile constituents (Debboun et al., 2006; Jacob et al., 2007) included in the pesticide consisting of not only the active ingredient but also inactive ones such as adjuvants and additives because the fresh pesticide usually includes more volatile constituents than the old one. Secondly, the disturbance of each colony due to our observation in the beehive causes a reduction in foraging activity and therefore that honeybees seem to directly ingest toxic sugar syrup more, which cannot be stored in cells on combs, than nontoxic nectar in fields gives rise to massive death of honeybees by a smaller amount of toxic sugar than in each interval. Thirdly, the pesticide rapidly ingested by honeybees just after the administration may cause a rapid weakening of their colony and the rapid loss of their appetite.

Except for the first interval after the first dinotefuran

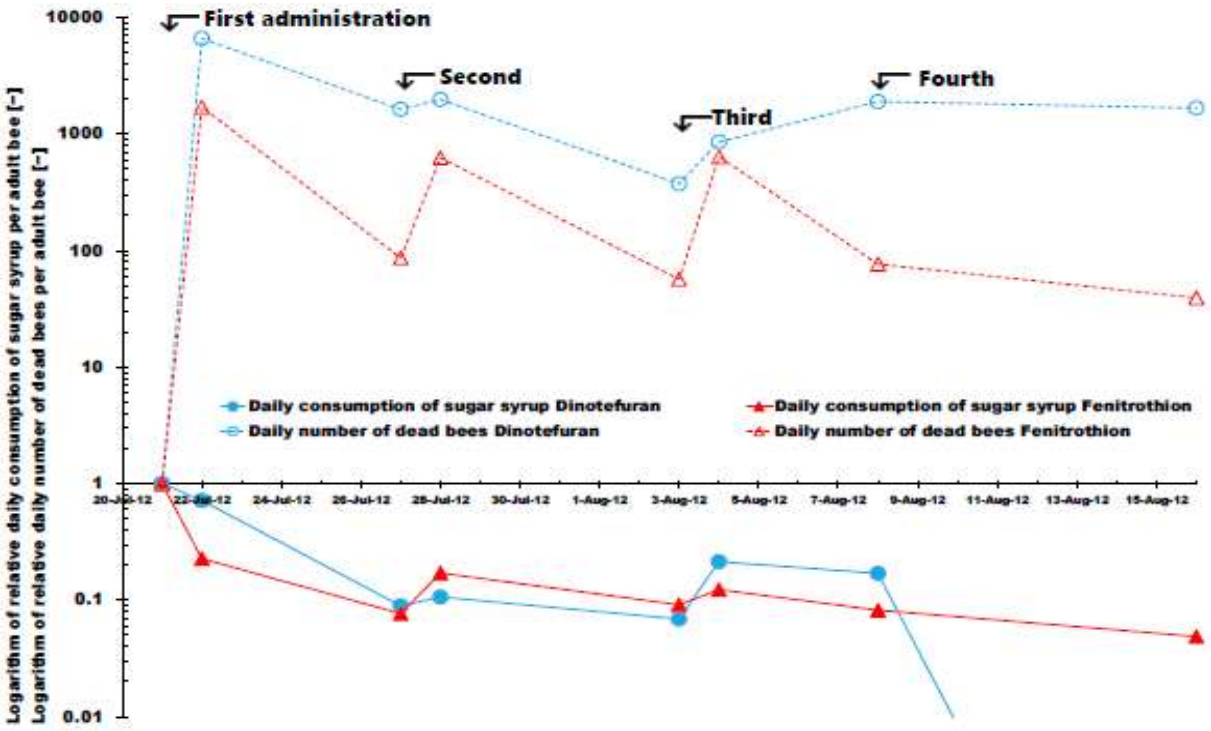


**Figure 9:** Daily consumption of sugar syrup per adult bee and daily number of dead bees per adult bee. The daily interval consumption of sugar syrup per adult bee [g/day/adult bee] is obtained from dividing the interval consumption of sugar syrup (Table 5) by the number of days in the interval between the two adjacent observation dates and by the average number of adult bees between two successive observation dates. The daily number of dead bees per adult bee (that is, mortality per day) [heads/day/adult bee] is obtained from dividing the number of dead bees in each interval shown in Table 2 by the number of days in the interval between two adjacent observation dates and by the average number of adult bees between the two successive observation dates. The relative values to a standard are shown in this figure, where the daily relative-number of dead bees is defined by an equation of {(the number of dead bees of experimental colony/ the number of days in an interval)/ the average number of adult bees of experimental colony between an interval}/ {(the number of dead bees of experimental colony in the interval between July 15$^{th}$ and 21$^{st}$/ the number of days (6 days) in the interval)/(the average number of adult bees of experimental colony between the interval}; similarly, the daily relative consumption of sugar syrup is defined by an equation of {(the consumption of sugar syrup of experimental colony in the interval/ the number of days in an interval)/ (the average number of adult bees of experimental colony in an interval / the number of days in an interval)} / {(the consumption of sugar syrup of control colony in the interval between July 21$^{th}$ and 22$^{st}$/ days of the interval (1 day)) /the average number of adult bees of control colony in the interval}. A standard of the daily consumption of sugar syrup per adult bee in assuming that each colony takes nontoxic sugar syrup is the average quantity of sugar syrup consumed by two control colonies for a day from July 21$^{st}$ to July 22$^{nd}$ as a substitute for the nontoxic quantity before the administration of the pesticide into each experimental colony because we have not measured the non-toxic quantity before the administration; 1000 g/ (9647+10133)/2=9891.5 heads for Control 1 (RUN1) and 1000 g/ (9665+9558)/2=9611.5heads for Control-2 (RUN4). A standard of the daily number of dead bee per adult bee for each experimental colony before the pesticide administration is obtained from dividing the number of dead bees measured on July 21$^{st}$ (5 heads for the dinotefuran colony; 3 heads for the fenitrothion one) by the number of days from July 15$^{th}$ to July 21$^{st}$ (6 days) and by the average value of the number of adult bees on July 15$^{th}$and that on July 21$^{st}$, that is, (6878+9173)/2=8025.5 heads for the dinotefuran colony (RUN2); similarly, (7565+8943)/2=8254 heads for the fenitrothion colony (RUN3). Their common logarithmic values are plotted except when they become zero. We assumed that the daily consumption of nontoxic (pesticide-free) sugar syrup per adult bee on July 21$^{st}$ before the administration of the pesticide seems to be almost the same as the average daily consumption of nontoxic sugar syrup per adult bee by the two control colonies (Control 1 and 2) from July 21$^{st}$ to July 22$^{nd}$, where the average value of two controls is 0.102570 g/day/adult bee; namely, 0.101097 g/day/adult bee in Control 1 and 0.104042 g/day/adult bee in Control 1 and 2. The dates in 2012 when the fresh pesticide through sugar syrup instead of old one was administered are as follows: The first pesticide administration date: July 21$^{st}$; the second date: July 27$^{th}$ and the third date: August 3$^{rd}$. The observations were subsequently conducted on the following day in order to investigate the effect of fresh pesticide sugar syrup on a honeybee colony.

administration, the daily consumption of toxic sugar syrup per bee by each experimental (dinotefuran, fenitrothion) colony rapidly decreased just after every administration of the pesticide gradually increased with time between the day after the administration date and just before the next administration when the daily consumption of toxic sugar

syrup reaches its peak. The saw-tooth-like change in the daily consumption of toxic sugar syrup is repeated thrice after the first, second and third administrations. After the fourth administration of fenitrothion, the saw-tooth-like change was not seen and continued to increase slightly and gradually. On the other hand, only in the interval between the day after the first administration and just before the second one, the daily consumption of toxic sugar syrup per adult bee by the dinotefuran colony gradually decreased with time contrary to the other cases. The exceptional gradual decrease in the daily consumption of toxic sugar syrup in the first interval by the dinotefuran colony seems to be due to the following reasons: Firstly, part of toxic sugar syrup consumed by the dinotefuran colony from July 21$^{st}$ to 22$^{nd}$ is directly ingested by honeybees and the rest stored in cells on combs in a beehive after conversion into toxic honey after the first administration. We can infer that as the stored toxic sugar syrup (honey) was continuously ingested by the dinotefuran colony after the first administration, the daily consumption slightly decreased with time after the first administration and just before the second administration.. Secondly, the dinotefuran colony can be enfeebled by a great deal of the intake of toxic sugar syrup with dinotefuran just after the first administration and therefore honeybees can lose their appetite.

The reason why the daily consumption of toxic sugar syrup by each experimental colony gradually increased with time in the interval between the day after the administration date and just before the next administration can be broadly explained with exception of the daily consumption of toxic sugar syrup in the first interval from July 21$^{st}$ to 22$^{nd}$ by the dinotefuran colony; Firstly, a decrease in volatile constituents included in the pesticide with time resulted in an increase in the consumption of toxic sugar syrup stored in cells on combs, considering also the facts that mosquitoes are able to ignore the smell of the insect repellent within a few hours of being exposed to it (Stanczyk et al., 2013) and organophosphates induced a phenomenon that was first attributed to the repellency to foraging bees (Belzunces et al., 2012). Secondly, as brood can take toxic sugar syrup less than adult bees, adult bees emerging from capped brood in each interval will consume more toxic sugar syrup than the other honeybees which already have ingested the pesticide because they can be more active than the others. In this case, the daily consumption of toxic sugar syrup will increase after a time lag.

Despite similar level of daily consumptions of both pesticides, the much higher level of the daily number of dead bees in the dinotefuran colony than the fenitorothion colony means that dinotefuran seems to be highly toxic for a honeybee than fenitrothion under the same insecticidal activity for a stinkbug.

Incidentally, we should consider that this consumption of toxic sugar syrup and number of dead bee per adult bee can contain some margin of error when the population to which the adult bees belong is small.

**Why does the fenitrothion colony succeed in overwintering?**

A significant difference was observed between the neonicotinoid dinotefuran and the organophosphate fenitrothion despite the same insecticidal activity for stinkbugs. The dinotefuran colony became rapidly extinct within a month, while the fenitrothion colony succeeded in overwintering notwithstanding that it had taken a substantial amount of toxic sugar syrup. It seems probable that easy decomposability and short-term persistence of fenitrothion can lead to a success in the fenitrothion colony overwinter and recovering it from the damages due to the organophosphate fenitrothion.

Here, we will examine whether damages to a honeybee colony that has been inflicted by a pesticide were recovered or not under the subsequent pesticide-free conditions in our previous long-term field experiments (Yamada et al., 2012, 2018). The colonies into which the neonicotinoids dinotefuran and clothianidin was administered had never been able to recover from the damages due to the pesticides even after both pesticides having one-tenth insecticidal activity to exterminate stinkbugs were administered only once and as such we converted from toxic foods (sugar syrup and pollen paste) to pesticide-free foods. This is probably attributed to the long-term persistence of neonicotinoids as reported by Yamada et al. (2012). In addition, we have the fact that the dinotefuran colony, where a low concentration of dinotefuran (0.565 ppm) was administered through pollen paste into which non-toxic pollen was kneaded with toxic sugar syrup having one-hundredth insecticidal activity to exterminate stinkbugs failed in overwintering at the intake of dinotefuran of about 61 ng/bee, as reported by Yamada et al. (2018), though it looked vigorous before winter. It can be deduced from these findings that neonicotinoids can cause not only a CCD but also a failure in overwintering.

**Difference in the survival period of the dinotefuran colony between this work and previous work (Yamada et al., 2012)**

The dinotefuran colony in this work led to the much more rapid extinction (26 days) than that (61 days) in previous work as reported by Yamada et al. (2012) under the same concentration. How such inconsistency could arise should be considered. Table 7 shows the cumulative total intake of dinotefuran per bee till the colony extinction in this work, in comparison with those in our previous works experimented in 2010 (Yamada et al., 2012) and in 2011 (Yamada et al., 2018). Figure 6 shows the comparison of the estimated cumulative total intake of dinotefuran per bee till the extinction of colony among our field-experimental results. It is observed from Figure 6 that there is a significant difference in the cumulative total intake of pesticide per bee

**Table 7:** Cumulative total intake of pesticide per bee till colony extinction (during the administration of pesticide).

| Pesticide | 2012 (DF-2 ppm) | 2011 (DF-1 ppm)[1)] | 2010 (DF-1 ppm)[2)] | 2010 (DF-2 ppm)[2)] | 2012 (FT-10 ppm) |
|---|---|---|---|---|---|
| | Dinotefuran | | | | Fenitrothion |
| Concentration of pesticide in vehicle | 2 ppm | 1 ppm | 1 ppm | 2 ppm | 10 ppm |
| Dilution factor against a concentration to exterminate stinkbugs | A fiftieth part to exterminate stinkbugs | A hundredth part to exterminate stinkbugs | A hundredth part to exterminate stinkbugs | A fiftieth part to exterminate stinkbugs | A fiftieth part to exterminate stinkbugs |
| Vehicle to administer the pesticide | Sugar syrup | Sugar syrup | Both sugar syrup and pollen paste | Both sugar syrup and pollen paste | Sugar syrup |
| Notation | DF-Middle | DF-Low | DF-Low | DF-Middle | FT-Middle |
| Cumulative total intake of the pesticide per bee till extinction [ng/bee] | 93.8 | 310.7 | 349.8 | 310.0 | 862.5 |
| Period to estimate the intake of pesticide | From start to colony extinction | From start to colony extinction | From start to colony extinction | From start to colony extinction | From start to stop of pesticide administration |

Yamada et al. (2012) under submission to Journal Apicultural Research. [1]: (Bal et al., 2013); [2]: (BASF, 2014). The fenitrothion colony (RUN3) in this work did not become extinct, the intake per bee was estimated using the cumulative number of honeybees and the cumulative total intake of fenitrothion taken by honeybees from the start of the pesticide administration on July 21st 2012 to the finish on August 16th 2012 when the dinotefuran colony (RUN2) in this work became extinct.

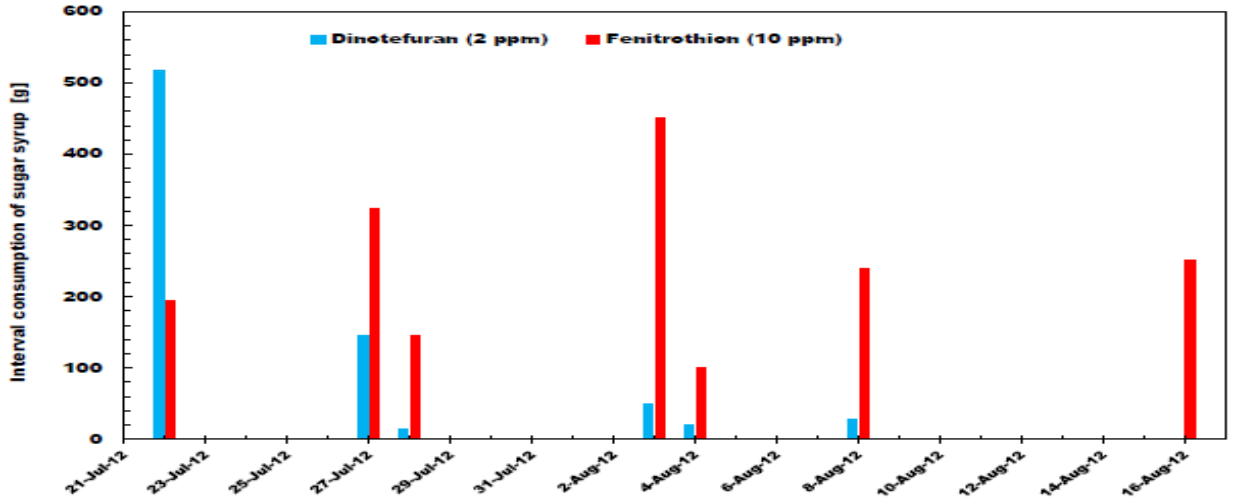


**Figure 10:** Interval consumption of sugar syrup with the pesticide for each colony two adjacent observation dates in this work. The interval intake of sugar syrup can be obtained by the amount of sugar syrup consumed by each colony between two adjacent observation dates: Ex. The interval intake on July 27th is the amount of sugar syrup consumed from July 22nd till 27th in a colony.

between this work and previous works. In this work conducted at the concentration of 2 ppm, we observed that more than half the initial number of honeybees died within a day after the first administration and the colony became extinct after the elapse of 26 days while a honeybee was estimated to take dinotefuran of 93.8 ng/bee. In previous work conducted at the concentration of 2 ppm in 2010 (Yamada et al., 2012), a number of dead bees occurred only in the early period after the start of administration but they almost never occurred afterwards and the colony became extinct after the elapse of 61 days while a honeybee was estimated to take dinotefuran of 310.0 ng/bee. In other previous works conducted at the concentration of 1 ppm in 2010 and 2011, dead bees almost never occurred after the administration and the colony became extinct after the elapses of 84 days in 2010 (Yamada et al., 2012) and 104 days in 2011 (Yamada et al., 2018), while a honeybee was estimated to take dinotefuran of 349.8 and 310.7 ng/bee, in 2011, respectively. The colony extinction in this work seems to be chiefly triggered by a massive death due to acute toxicity, while the extinction in previous works seems to be caused by chronic toxicity with an aspect of a CCD on assumption.

**Why did the dinotefuran colony in this work become extinct by assuming an aspect of acute toxicity?**

At present, the reason why the dinotefuran colony in this work became extinct after surviving for only 26 days probably due to acute toxicity earlier than that in our previous work (Yamada et al., 2012) which had become extinct after surviving for 61 days probably due to chronic toxicity under almost similar concentrations of dinotefuran was deduced. In the field experiment of an actual apiary, all of toxic sugar syrup with dinotefuran that is administered is not taken instantly, but stored as honey and the excipient of bee bread after the toxic sugar syrup has been mixed by nectar or pollen without pesticides gathered from fields and the toxicity attenuated. Considering that the amount and pesticide-concentration of toxic sugar syrup stored depend on the weather and/or the blooming season (Tesfay, 2007; Gebremedhn et al., 2014), we will discuss the weather conditions near the experimental site in the region (Noto District, Ishikawa Prefecture, Japan) where we conducted the experiment in our apiary. We cannot find the difference in blooming season between the previous work and this. Thereafter, we carefully investigated the weather for the initial period after toxic sugar syrup with dinotefuran was administered into a honeybee colony because the initial intake of the pesticide (dinotefuran) seems to affect majorly the honeybee colony.

Here, we examined the changes in maximum atmospheric temperatures of the days for about a month from the middle of July to the beginning of August in 2010 and 2012 in Noto District near the experimental site based on weather data archived by The Japan Weather Association. Comparing the changes in maximum atmospheric temperatures of the days for a month between 2010 (Yamada et al., 2012) and 2012 (this work) as shown in Figure 11, we can find that there was a significant difference between them for a week around the start of experiment. Examining a maximum atmospheric temperature of each day from three days before the start of experiment to three days after, we can find the fact that the maximum, minimum and average among them are 34, 27 and 31.5°C in 2010; and 30, 27 and 28.3°C in 2012, respectively. The maximum, minimum and average of atmospheric temperatures for two weeks after the start of experiment were 34, 31 and 32.4°C in 2010; and 36, 27 and 32.3°C in 2012, respectively. We observed the temperatures just after the start of the experiment in 2012 (their average of 28.3°C) are lower than those in 2010 (their average being 31.5°C). The difference in temperature change between the two will be further discussed.

Generally, the foraging activity (flight intensity) of honeybees tends to increase with temperature (Tesfay, 2007; Gebremedhn et al., 2014). According to Tesfay (2007), the number of honeybees visiting sunflower inflorescences during peak flowering when atmospheric temperature ranges from about 25 to 35°C changes with temperature while passing through three phases as follows: First, the foraging activity of honeybees (the number of honeybees visiting sunflower inflorescences) increases sharply from about 25 to about 30°C. Second, it takes a maximum value at about 30°C and then the maximum value is maintained till about 32°C Thirdly, after that it begins to decrease. Judging from the findings obtained by Tesfay (2007) and the temperature changes in our experimental site (Noto District in Japan), the foraging activity seems to remain high because the maximum temperatures ranged from 31 to 34°C in 2010, but it seems to be fairly low for a few days just after the first administration of pesticide (dinotefuran) in 2012. Besides, the wide fluctuation of the temperatures from 27 to 36°C in 2012 which take sometimes a value lower than 30°C or higher than 35°C will probably lead to a further decrease in foraging activity. When the foraging activity is low, it will be generally accepted that honeybees bring less foods (nectar and pollen) from fields. From the aforementioned difference of the foraging activity due to atmospheric temperatures between the experiment conducted in 2010 (Yamada et al., 2012) and that in 2012 (this work), it can be inferred that the dinotefuran colony in 2010 (Yamada et al., 2012) could bring more non-toxic foods from our pesticide-free fields proximate to the experimental site and could directly ingest less toxic foods administered to a beehive in the experiment than that in 2012 (this work), because the foraging activity in 2010 would be higher than that in 2012 in the early period of the start of the pesticide administration. In the 2012 experiment (this work) which started in the period when such atmospheric temperatures bring lower foraging activity and the blooming season had passed, the colony having the lower foraging activity in 2012 directly ingested

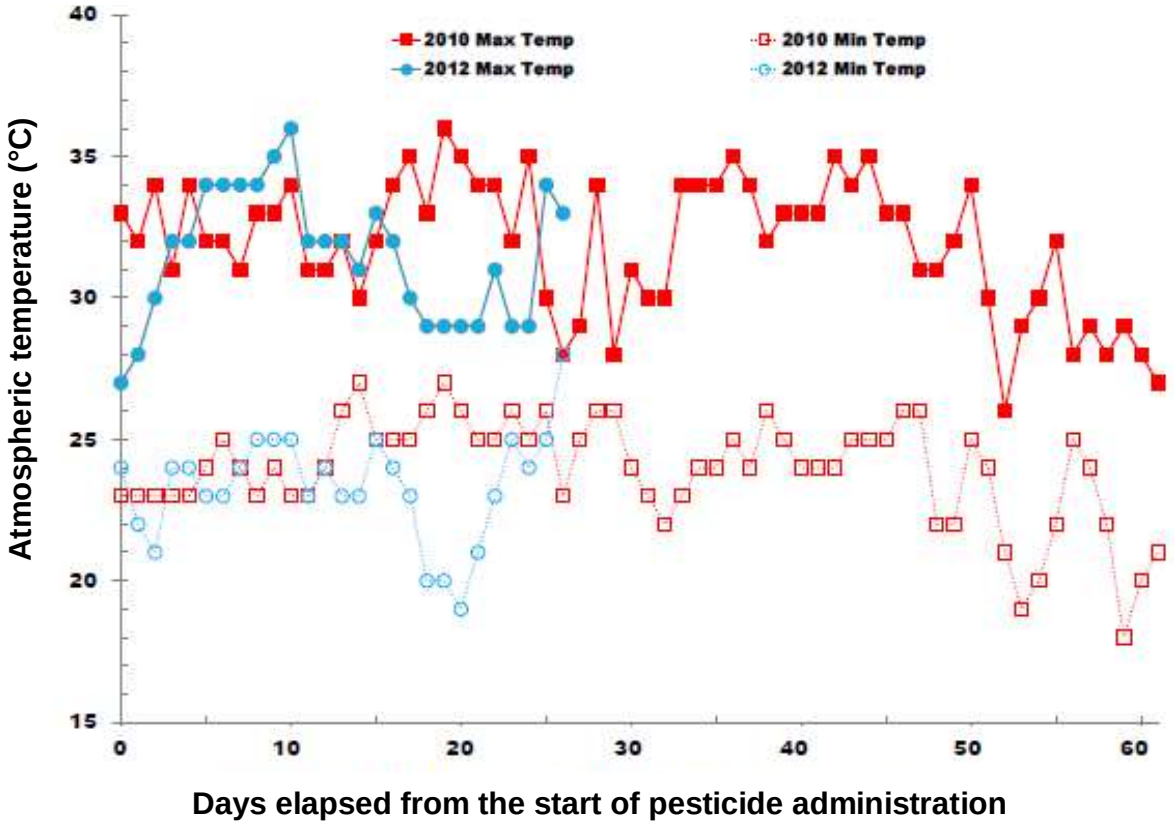


**Figure 11:** Changes in atmospheric temperature in Noto District in Japan near the experimental site between 2010 and 2012. The atmospheric temperatures are cited from the archived data by The Japan Weather Association.

more toxic sugar syrup and was more susceptible to an acute toxicity than the colony having the higher foraging activity in comparison with the 2010 experiment (Yamada et al., 2012) which started in the period when the temperatures bring higher foraging activity and the blooming season was still on. We paid attention to the fact that honeybees will preferentially take foods (nectar and pollen) from fields to a substitute such as sugar syrup and powdered soy bean and the leftover substitute which cannot be directly ingested will be preferentially stored in cells on combs.

It will be generally accepted that honeybees seem to prefer natural foods (nectar and pollen) to artificial foods (sugar syrup and pollen substitute) and they prefer non-toxic foods to toxic foods. From the aforementioned, we can infer that honeybees ingested foods in which a ratio of natural and non-toxic foods from our apiary to artificial toxic foods is higher in previous work in 2010 than in this work in 2012 and the intake of dinotefuran from ingested foods is less in our previous work in 2010 than in this work in 2012. We may also infer that foods (honey and bee bread) stored in the colony in this work (conducted in 2012) becomes less than that in previous work conducted in 2010 and the concentration of pesticide (dinotefuran) in stored foods in this work will become higher than that in our previous work though the pesticide concentration of sugar syrup stored in cells naturally becomes lower by the foods taken from our pesticide-free apiary than the original concentration whenever experimental toxic foods (sugar syrup and pollen paste) are stored in cells. Honeybees actually ingested more pesticide (dinotefuran) and the colony became extinct in a shorter period of time after the first administration of pesticide assuming an aspect of acute toxicity in this work than in our previous work which had assumed an aspect of a CCD, while the intake of dinotefuran per bee in this work was apparently less than that in our previous work

On the other hand, we deduced that the main reason for few differences in the intake of dinotefuran per bee between the experimental results in 2010 and those in 2011 can come from few difference of change in atmospheric temperature between the two as reported previously (Yamada et al., 2012, 2018). The difference in atmospheric temperature changes may probably cause difference in the survival period of a colony as it earlier described that the colony became extinct earlier in this work than that in our previous work. Here, we should perceive in an experimental apiary that the entire amount of pesticide administered into a colony through food is not instantly taken by honeybees, and that some amount of the pesticide can be stored in cells on combs after mixed with foods imported from fields where pesticides may or may not exist. In order to obtain the

amount of the pesticide stored in the beehive (combs), it may be necessary to accurately determine the amounts of honey and bee bread in each comb and the concentration of the pesticide in them in every observation. In this work we have reluctantly decided to relinquish their measurement which may approach the impossible because of a great deal of expenses and labor though it is desirable that our deductive inferences earlier mentioned would be substantiated by new facts.

**Why is the intake of dinotefuran per bee till colony extinction in this work less than that in our previous ones?**

The difference in the survival period of the dinotefuran colony between this work and the previous work was earlier discussed (Yamada et al., 2012). Here, we discussed the reason why there is a vast difference in the intake of dinotefuran per bee till the extinction of colony between the experiment conducted in 2012 (this work) and that in 2010 (Yamada et al., 2010), though both experiments were conducted with similar concentrations of dinotefuran as shown in Table 7 and Figure 6. We can deduce the reason for the difference in the intake till the colony extinction between 93.8 ng/bee in 2012 and 310 ng/bee in 2010 from the viewpoint of the foraging activity due to the weather as follows:

As previously discussed, the dinotefuran colony in the 2010 experiment (Yamada et al., 2012) seems to have directly ingested less toxic food and stored more toxic food in cells on combs by house bees which work independently on weather than that in the 2012 experiment (this work) according to the deduction from the foraging activity due to the weather. Figures 7 and 8 show the cumulative intake of pesticides taken by a honeybee till a certain observation date in our previous work conducted in 2010 (Yamada et al., 2012) and that in this work conducted in 2012, respectively. The cumulative intake of pesticide per bee can be obtained from dividing the cumulative intake of pesticide per a colony till a certain observation date by the cumulative number of honeybees which is given by the sum of both the initial number of adult bees at the start of experiment and the number of newborn bees till a certain observation date. Comparing these curves of dinotefuran between in 2010 (Figure 7) and in 2012 (Figure 8), we can find that the cumulative intake of dinotefuran in 2012 (this work) rapidly increases at the start of the experiment but that in 2010 (previous work) gradually increased. This fact can sustain the aforementioned presumption that higher foraging activity deeply depends on weather results in lower intake of toxic food experimentally fed to a colony.

**Can the $LD_{50}$ assess the impact of a pesticide sprayed in fields on a honeybee colony in an apiary?**

The $LD_{50}$ is well-known as an indicator for acute toxicity of pesticides. The $LD_{50}$ for honeybees is defined by the amount of pesticide individually taken forcefully and kills half of the honeybees within a limited time. The various values of the $LD_{50}$ for fenitrothion was reported by US-EPA (1995) (20 ng/bee for contact; 380 ng/bee for contact), (Wang et al. 2012) (30 to 40 ng/bee for contact), (Takeuchi et al., 1980) (130 ng/bee for contact), (Okada and Hoshiba, 1970) (30 ng/bee for contact), (NUFARMNZ, 2012) (18 ng/bee), (University of Hertfordshire, 2013) (160 ng/bee for contact), (Sanford, 2003) (176 ng/bee for contact) and (WHO, 2010) (200 ng/bee for acute oral and 160 ng/bee for acute contact). The various $LD_{50}$ values for dinotefuran were also reported by US-EPA (2004) (23 ng/bee for acute oral and 47 ng/bee for contact); (32 ng/bee for acute oral and 61 ng/bee for contact); (7.6 ng/bee for acute oral and 24 ng/bee for contact); (Iwasa et al., 2004) (75 ng/bee for contact) and Durkin (2009) (47 ng/bee for acute contact).

The $LD_{50}$ is measured in the laboratory under controlled conditions, but in an actual apiary such as this field experiment site, there are many uncontrollable factors such as the behavior of a honeybee as a member of a colony and environmental conditions, such as the weather, etc. Uncontrollable factors of environmental conditions and the weather can be cancelled to a certain degree by control experiment. Judging from these $LD_{50}$, the intake of the pesticide per bee as shown in our works are so high that the colony should be naturally expected to become extinct instantly. Above all it is not understandable from the $LD_{50}$ why the fenitrothion colony (RUN3) could even succeed in overwintering despite the fact that the intake of fenitrothion per bee was much higher than the $LD_{50}$. One of the possible causes is that the ingestion of a pesticide which is administered into a beehive is not compulsory in the field experiment. The second is the stored toxic sugar syrup in cells on combs, which was diluted by pesticide-free honey from organic fields. This will be applicable to the dinotefuran colony (RUN2) because it continued to survive for 26 days while the cumulative total pesticide intake is enough to exterminate the colony within a few days.

In field conditions, a honey bee is free to go wherever she wants and take food whenever she wants, thereafter, she can selectively take food from fields if she prefer food in fields, which is unknown be it toxic or non-toxic to toxic food with a pesticide administered. At a concentration of 2 ppm of dinotefuran in sugar syrup in this work, honeybees seem to be alive for a little while after the intake of the pesticide. While they are alive, they can convert toxic sugar syrup that they have taken from a feeder into toxic honey and can temporally store it in cells on combs.

Toxic honey can be mixed with honey made from nectar in fields when it is stored in a cell or toxic sugar syrup can be mixed with nectar gathered from fields in honeybees' bodies. Through a series of these processes, the toxicity of honey can be diluted when it is stored in a cell. After pollen is kneaded with toxic honey to be bee-bread, it is stored in cells on combs. In this work, nectar and pollen from fields seems to be non-toxic because we have regulated our apiary to be

pesticide-free, though, there is a slight possibility that nectar and pollen may be collected from fields where pesticides are not controlled other than our apiary.

The foods stored (honey, bee bread) are consumed by adult bees, brood and queens. The food containing neonicotinoids such as dinotefuran continue to adversely affect a honeybee colony for a prolonged period of time but the food containing organophosphates does not affect a colony over a prolonged period because organophosphates such as fenitrothion can be easily decomposed and become non-toxic. It can be deduced that the difference in persistence between organophosphates such as fenitrothion and neonicotinoids such as dinotefuran leads to a difference between success and failure in overwintering based on the fact that the fenitrothion colony in this work succeeded in overwintering but the dinotefuran colonies in both previous work (Yamada et al., 2018) and this work failed in overwintering though it looked vigorous before winter.

Besides the earlier mentioned reasons why the $LD_{50}$ cannot assess the impact of a pesticide sprayed in fields on a honeybee colony in an apiary, we have to consider that the $LD_{50}$ cannot always give toxicological evaluations for a colony of honeybees which are eusocial insects because it can only be used to assess an individual living creature. We strongly desire a new indicator to assess chronic toxicity for a honeybee colony instead of the $LD_{50}$.

**How could a CCD possibly be caused by a pesticide in an actual apiary?**

It is defined as a CCD that a honeybee colony exhibit all the following symptoms; a colony's worker bee population is suddenly lost with very few dead bees found near the colony; the queen and brood remained; and the colonies had relatively abundant honey and pollen reserves; finally, the colony cannot sustain itself without worker bees and would eventually die.

We considered some convincing stories on a change in the state of a honeybee colony which has taken a pesticide in an actual apiary based on the findings obtained from the long-term field experiments. Principally, the process which the colony undergoes when the colony assumes an aspect of a CCD will be discussed.

When a pesticide is sprayed in fields, many foraging bees which are directly exposed to its high toxicity are instantly killed on the spot due to the decrease in the caretakers and acute toxicity and the colony becomes short of foraging bees. Some house bees are recruited as foraging bees and the caretakers of the brood become shorthanded in the colony. The queen lays fewer eggs due to toxicity of a long-term persistent pesticide such as dinotefuran though a short-term persistent pesticide such as fenitrothion seems to affect the long-lived queen restrictively and slightly. The colony dwindles away while becoming weakened and more susceptible to attacks by pests and pathogens. Finally, the colony cannot sustain itself and it collapses or escapes from the beehive. The colony which has taken a short-term persistent pesticide can sometimes survive.

When the toxicity and/or concentration of a pesticide is not so high, many foraging bees which are killed on the spot contaminated by the pesticide, can bring toxic water, toxic foods (pollen and nectar) back to their beehive. House bees directly ingest some of them or store the leftover in cells on combs after the toxic foods are diluted with non-toxic foods foraged from other uncontaminated fields or are mixed with toxic foods imported from the other fields contaminated by pesticides. Some of the honeybees exposed to a pesticide in the beehive are occasionally killed in a short time due to acute toxicity and others become weakened or get lost in fields depending on the amount of the pesticide taken by them. The stored toxic foods continue to affect the colony adversely for a long period of time due to chronic toxicity if the pesticide is persistent. Exposure to long-term chronic toxicity will weaken not only adult bees and brood but also the queen will decrease in queen's ovipositional performance, cause the disorientation of foraging bees, lead to the breakdown of polyethism in the colony and also threaten the colony to extinction while few dead bees are found around the beehive. In this case, a CCD can occur. The CCD phenomenon will be probably caused by the chronic toxicity of a long-term persistent pesticide such as a neonicotinoid which continues to have an enduring effect on a honeybee colony.

When most of foraging bees are not directly exposed to a pesticide, they will take toxic water, toxic pollen and toxic nectar in fields where the pesticide is sprayed while their toxicity is weakened by dilution with rainwater if the pesticide is water-soluble (systemic) and/or by degradation due to sunlight. Foraging bees bring foods (water, pollen and nectar) whose toxicity is weakened back to their beehive and honeybees store some of the foods in cells on combs in the colony after the toxicity of the foods is changed by foods foraged from other fields. Honeybees become weakened and get lost in fields due to chronic toxicity. The amount of toxic foods stored in cells on combs depends on the foraging activity which is strongly influenced by environmental conditions such as weather and blooming conditions as can be seen from the difference in pesticide intake between this work and previous work (Yamada et al., 2012) under similar experimental conditions. Moreover, toxic water near fields contaminated with the pesticide also continues to adversely affect the colony while the toxicity is diluted with rainwater if the pesticide is persistent and highly toxic. The stored foods and toxic water in fields continue to adversely affect the colony for a long period of time chronically even when the pesticide is sprayed long time ago in fields, if the pesticide is persistent. In this case, a CCD can occur. Even if the toxicity of long-term persistent pesticide is too low to cause a CCD during an active period of honeybees, it can cause failure in overwintering due to chronic toxicity in cases where the colony looks vigorous before winter. The

reason is that honeybees continue to ingest only toxic foods, which are stored before winter.

We can infer that the disasters to a honeybee colony such as CCD, wintering loss and massive death seem to be caused by the synergy effects due to a combination of the characteristics of a neonicotinoid pesticide such as long-term persistence, systemic property and high toxicity. The long-term persistence of a neonicotinoid permits a pesticide to maintain its toxicity for long periods of time under the natural environment. For examples, it permits its toxicity to be kept in foods stored in cells on combs for long and also the environment to be contaminated with a pesticide by the wide and prolonged diffusion of the toxicity of the pesticide dissolving in water in fields. The systemic property permits a pesticide to dissolve easily in water and to be of wide distribution over the whole plant. The high toxicity permits it to prolong its toxicity for a longer period of time even after it is diluted by large quantities of rain water. On the other hand, an organophosphate seems hard to cause such disasters except massive death just after being sprayed because it is probably much less persistent and less toxic than a neonicotinoid.

**A maximum concentration of a pesticide in nectar which a foraging bee can bring back to her colony from a field**

Here, we estimated the amount of concentration of a pesticide that can cause instant death of foraging bees in fields and makes foraging bees unable to return to their beehive. A foraging bee has a honey stomach in which she can store 18 to 77 mg of nectar (Cooper et al., 1985) and can carry about 40 mg of nectar (Yadav, 2003). The consumption of nectar per flight is about 13 mg under the assumption that the consumption of a foraging bee can be an approximate equivalent of the consumption of a drone (Burgett, 1973). When the pesticide concentration of nectar is x ppm, a foraging bee may carry 40x ng of a pesticide per flight and may take 13x ng of a pesticide during flight. Here, a pesticide seems to act as a contact toxicity stored in the honey stomach of a foraging bee and ingested during transport.

Currently, we considered the case where foraging bee carry toxic nectar contaminated with dinotefuran to her colony from fields where dinotefuran has been sprayed and assumed that the $LD_{50}$ of dinotefuran is about 23 ng/bee for oral or about 61 ng/bee for contact (US-EPA, 2004) and most of the foraging bees may die instantly on the spot at about twice the intake of a pesticide as much as the $LD_{50}$.

In the case earlier mentioned, we can obtain the threshold of dinotefuran concentration beyond which a foraging bee will die during transportation and not be able to carry toxic nectar back to her colony (beehive) as follows: Assuming that a foraging bee consumes 13 mg of nectar during transportation (Burgett, 1973) and she dies at the intake of more than twice of $LD_{50}$, we can obtain the threshold nectar concentration of about 3 ppm due to contact in honey stomach from the relation of 2×LD50 (contact) / amount of nectar in honey stomach = 2×61/40≈3 and that of 3.5 ppm due to oral ingestion during transportation from the relation of 2×LD50 (oral) / ingested amount of nectar during transportation = 2×23/13≈3.5. That is, most of foraging bees which visit the field contaminated by dinotefuran of 3 ppm or more can probably be killed outright or during transportation and cannot return to their beehive (colony).

A maximum concentration of dinotefuran in honey stored in a cell on a comb can be estimated to be about 12 ppm as dinotefuran in nectar concentrated four times assuming that a water content in nectar is 80%, that in honey is 20% and a concentration of dinotefuran in nectar is 3 ppm. It can be deduced from the aforementioned estimation that honeybees can store honey with extremely high concentrations of pesticides as compared with the $LD_{50}$ for honeybee.

**Differences in impact on a honeybee colony between dinotefuran and fenitrothion**

Although we prepared toxic sugar syrup with both the concentration of dinotefuran and that of fenitrothion having one-fiftieth insecticidal activity to exterminate stinkbugs on the assumption that a pesticide was sprayed in the neighborhood and we obtained very different results on the colony between the two pesticides as follows:

(1) The neonicotinoid dinotefuran colony (RUN2) became extinct after the elapse of 26 days from the administration of the pesticide but the organophosphate fenitrothion colony (RUN3) did not become extinct and even succeeded in overwintering (Figures 3 and 4 and Table 4).
(2) Nearly half the initial adult bees in the dinotefuran colony were killed within a day after the administration of the pesticide dinotefuran (from July 21$^{st}$ to 22$^{nd}$), but only about one-tenth of the initial adult bees in the fenitrothion colony were killed just after the administration of the pesticide fenitrothion, though both pesticide concentrations were prepared so as to be identical in insecticidal activity for stinkbugs. A great difference in the initial mortality of adult bees between the dinotefuran colony (4838/9173≈0.527) and the fenitrothion (865/8943≈0.097) colony may come from the difference in the initial intake of toxic sugar syrup which is more in the dinotefuran colony (518 g) than in the fenitrothion colony (195 g) (Figure 5 and Table 5).
(3) On the other hand, capped brood in both colonies cannot decrease very much just after the pesticide administration. This indicates that it will take some amount of time for both pesticides to affect capped brood because eggs and larvae which are apt to ingest a pesticide turn into capped brood which can hardly ingest it.
(4) Adult bees and capped brood decrease in number with the elapse of time in both dinotefuran and fenitrothion colonies, but both rates of decrease in the dinotefuran colony

are higher than those in the fenitrothion. The difference in their rates of decrease over time between two experimental colonies can be attributed to the difference in persistence between both pesticides.

(5) The fenitrothion colony had a peak of the number of dead bees per day on the first day after each administration date (July 22nd, 28th and August 4th) more clearly than the dinotefuran colony which had an extremely high peak only once on the first day after the first administration date (July 22nd) (Figure 2 and Table 3). This fact suggests that fenitrothion will be easy to decompose in a short period of time and consequently its toxicity will last only a short time and become low sooner.

## Conclusion

According to the field experiment conducted from the end of June, 2012 to the middle of May, 2013, we confirmed that dinotefuran has much longer persistence on the honeybee colony in the field in comparison with fenitrothion. Although the concentrations of dinotefuran and fenitrothion were adjusted to affect an individual bee on the same level as each other in terms of the $LD_{50}$, there were clear differences between the dinotefuran colony and the fenitothion colony as follows: The dinotefuran colony became extinct within a month while the fenitrothion colony succeeded even in overwintering instead of colony extinction.

Our findings seem to throw light on the persistent effects of pesticides in the field that cannot be estimated only from the $LD_{50}$ which will be an indicator of the acute toxicity of a pesticide to an individual under laboratory conditions. The fenitrothion colony is estimated to have taken enough amount of the pesticide to be extinct from the viewpoint of the short-term effects. During the administration, a bee in the fenitrothion colony is estimated to have taken 862.5 ng/bee of fenitrothion that is 4.3 or more times larger than the $LD_{50}$ for acute oral (for example, 200 ng/bee) as reported by WHO (2010). The ratio of intake per bee to the $LD_{50}$ of fenitrothion is comparable with that of dinotefran. Accordingly, the fenitrothion colony should be extinct at almost the same time as the dinotefran colony if the $LD_{50}$ can precisely evaluate the influence of all kinds of pesticides. Making an assessment of persistence of pesticides is urgent for the precise evaluation of the persistent toxicity to the wild animals and insects. To make an assessment, we need to pay more attention to a complicated phenomenon itself, which tends to be overlooked in laboratory experiments in the natural environment.

We observed that there was a significant difference in the impact on adult bees between dinotefuran and fenitrothion. Dinotefuran caused a decrease in the number of adult bees in the colony about thrice faster than fenitrothion though both pesticides wield roughly equal influence on capped brood. Therefore, we infer that the extinction of the dinotefuran colony was attributed to a breakdown in division of labor due to the rapid unbalancing of the number of worker bees in a colony.

We speculated about the following negative influence of neonicotinoids on honeybee colonies in the natural environment based on our field experimental results. Since a neonicotinoid is a tasteless, scentless and persistent pesticide, honeybees continue to take it for a long time from water in fields. For instance, a rice paddy is one of the typical water resources for honeybees in Japan. A neonicotinoid pesticide sprayed in a rice paddy rapidly dissolves in water and widely diffused through water. As a neonicotinoid is of long-persistence, its toxicity is maintained in a rice paddy for a long period of time. Honeybees therefore continue to take toxic water containing a persistent neonicotinoid from a rice paddy and the toxic water adversely affect their colony for a prolonged period of time. Since a persistent neonicotinoid is accumulated in the body of a honeybee even if its concentration is much lower than that of our experiments, it influences in particular an elder worker bee which takes more toxic water for a longer period of time and causes a collapse of the colony maintained by the worker bees.

On the other hand, an organophosphate pesticide is unstable and not persistent in toxicity, which may lead to a rapid decay of toxicity with time. As an organophosphate pesticide which is sprayed in a rice paddy becomes non-toxic within a short period of time, its toxicity hardly influences a honeybee colony for a long period of time except just after the pesticide is sprayed. An organophosphate sprayed in a rice paddy could hardly cause serious problem such as collapse of a colony.

In this experiment, we cannot observe typical CCD phenomenon, which seems to be caused by chronic toxicity and is easily caused by a neonicotinoud in both dinotefuran and fenitrothion colony. Although the dinotefuran colony became extinct, many dead bees were found near the beehive just after the administration of dinotefuran. The existence of many dead bees cannot satisfy the requirements necessary to be recognized as a CCD phenomenon. Other aspects of a CCD such as the existence of the queen, capped broods and enough foods in the dinotefuran colony just before the colony extinction were observed. This result suggests that dinotefuran may or may not cause a CCD phenomenon by environmental conditions and the number of each member which takes charge of a job in the polyethism, even though the experiments were conducted at the same concentrations. On the other hand, far from becoming extinct, the fenitrothion colony restored itself and succeeded in overwintering after the discontinuation of fenitrothion administration, though, the colony was assumed to have already consumed more amount of fenitrothion per bee than enough to collapse judging from $LD_{50}$.

It may fairly be presumed from this that the success in overwintering of the fenitrothion colony is due to its short-persistence and fenitrothion is hard to cause a CCD phenomenon. These findings from a physiological point of

view can suggest that a CCD phenomenon will not be mysterious in a honeybee colony, but it will be only the final phase where a honeybee colony exposed by a persistent pesticide such as a neonicotinoid is gradually becoming extinct due to its chronic toxicity as already deduced by Yamada et al. (2012).

## ACKNOWLEDGMENTS

The authors would like to appreciate the effort of Ms. Hiroko Nakamura who helped devotedly to count accurately the numbers of adult bees and brood and also Mr. Yoshiki Nagai, Nanosystem, Inc., Japan for the development and improvement of the software used in counting automatically the numbers of adult bees and capped brood. Anonymous reviewer's insightful comments and suggestions are also acknowledged. We would like to specially thank Dr. Ken Hashimoto, Yamada Bee Farm, Inc., for his assistance and guidance offered in the course of this study and all the individuals that gave their assistance in order to make this research a success. This research was supported in part by Yamada Research Grant [grant number 74].

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**Cite this article as:**

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

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