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Research Paper

Comparison of the long-term influence of a pesticide on a bee colony between neonicotinoids (dinotefuran, clothianidin) and organophophate (fenitrothion) in Maui where there are neither harmful mites nor cold winter

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ABSTRACT

Four long-term field experiments were conducted on the impact of neonicotinoid pesticide (neonicotinoid) and organophosphate pesticide (organophosphate) on a honeybee colony in Japan. A few researchers in Japan assert that the chief culprit of a massive colony loss is not a neonicotinoid but an infestation of mites against our findings that a neonicotinoid would be deeply involved in the massive colony loss judging from the fact that mites and wax moth larvae cannot exist before colony extinction. We inferred from the field experiments in Japan that even when a colony does not collapse and looks active, a neonicotinoid can cause an egg-laying impediment of a queen and a decrease in immune strength of bees leading to the infestation of mites in a colony. In this work, we conducted a long-term (271 days) field experiment in Maui where there are neither mites harmful to honeybees nor distinct seasonal changes in organically-grown circumstances from October 22nd 2014 to July 20th 2015 with twelve colonies divided into four groups; each group consists of three colonies: a dinotefuran colony group where dinotefuran of 0.2 ppm was administered, a clothianidin colony group where clothianidin of 0.08 ppm was administered, a fenitrothion colony group where fenitrothion of 1 ppm was administered and finally, a control colony group where no pesticide was administered. These concentrations in the experiment conducted in Maui are same as those in the previous experiment conducted in Japan and are one-tenth of those in the other previous experiment. The results of the experiments in Japan where there are mites have been nearly duplicated on the experiment in Maui where there were no mites. In this work, the extinction ratios of neonicotinoid colony groups (dinotefuran and clothianidin) were 100% but those of both fenitrothion colony group and control colony group were 33.3%. From the long-term field experiments in both Japan and Maui, we can deduce the following conclusions: Neonicotinoid pesticides exterminate honeybee colonies with much higher probability than organophosphate pesticides which have the same extinction probability as control colonies have.

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Key words: Dinotefuran, clothianidin, fenitrothion, neonicotinoid, organophosphate, sugar syrup, field experiment, long-term, pesticide, honeybee, *Apis mellifera*, colony, overwintering, colony extinction, collapse, acute toxicity, chronic toxicity, Maui, wintering, mite, mite-free, seasonless.

INTRODUCTION

A neonicotinoid pesticide was suspected of being the cause of a massive death of honeybees about 20 years ago in France. After that various theories were proposed on the massive death. Many studies were occasioned by a massive loss of bee colonies due to a massive death of bees and/or a wintering failure in the Northern Hemisphere about a decade ago to find the causal agent (Steinhauer et al., 2015; Brodschneider et al., 2016; Kulhanek et al., 2017).

In recent years, it has been numerously reported that neonicotinoids pose a serious threat to ecosystems such as bee-colonies (Tirado, 2013; van Lexmond et al., 2015). There was an increasingly vocal demand that a field experiment was strongly desired to clear the phenomenon occurring in an apiary and the natural environment. At that time the massive death of honeybees was becoming serious among bee-keepers in Japan. We prepared a long-term field experiment in 2009 and conducted the experiment in 2010 in our apiary to reveal the effect of a neonicotinoid pesticide on a bee colony and concluded that a theory that a neonicotinoid pesticide was the cause of the massive losses would be most convincing (Yamada et al., 2012). Strangely enough, Lu et al. (2012) also conducted a similar field experiment in apiaries around this same time frame. Both studies demonstrated that a neonicotinoid pesticide adversely affected a honeybee colony and caused the colony extinction or failure in wintering.

Yamada et al. (2012) deduced that a Colony Collapse Disorder (CCD) will be possibly caused by the long persistency and high toxicity of a neonicotinoid pesticide and it will be only a scene where a bee colony is becoming extinct under long-term exposure to a neonicotinoid rather than a mysterious phenomenon. We, thereafter, conducted three long-term field experiments and (Yamada et al., 2018a) developed a new fact that a bee colony where the neonicotinoid dinotefuran was administered through pollen paste as a vehicle became extinct by about one-fifth of the dinotefuran intake of a colony where it was administered through sugar syrup as a vehicle and inferred that the difference in lethal intake between two vehicles of sugar syrup and pollen paste came from the difference in subject between adult bees which mainly ingested sugar syrup as an energy source and brood (queen) which mainly ingested pollen paste as a protein source. One of the key findings of Yamada et al. (2018b) was that a bee colony into which the neonicotinoid dinotefuran was administered became extinct as well as, our previous works (Yamada et al., 2012, 2018a) but a bee colony where the organophosphate fenitrothion was administered never became extinct and even succeeded in overwintering as well as, a control colony.

A concentration of a pesticide in our field experiments was determined based on the insecticidal activity of each pesticide to exterminate stinkbugs, judging from the facts in Japan that clothianidin with about 5 ppm concentration was detected in water around paddy fields (Kakuta et al., 2011) and the Maximum Residue Limits (MRLs) of clothianidin and dinotefuran in foods in Japan (JFCRF, 2017) are 1 and 2 ppm, respectively for rice (brown rice); 25 and 50 ppm for tea; 20 and 25 ppm for lettuce; 40 and 15 ppm for spinach; 3 and 2 ppm for tomato and 5 and 15 ppm for grape, respectively, as shown in Table 1.

It was pointed out by few researchers in Japan that the pesticide concentrations in our experiments were too high to be realistic in an apiary. We, therefore, conducted the field experiment under one-five hundredth of each recommended pesticide concentration to exterminate stinkbugs, that is, dinotefuran of 0.2 ppm, clothianidin of 0.08 ppm and 1 ppm of fenitrothion or 1 ppm of malathion (Yamada et al., 2018c). Yamada et al. (2018c) confirmed that a neonicotinoid (dinotefuran and clothianidin) collapse every bee colony as in the case of our previous experiments and it much more easily collapsed a bee colony than an organophosphate (fenitrothion and malathion) which seemed to affect a colony only about the same level with a control colony in wintering.

In that experiment, organophosphate residues could not be detected from honey in cells on frames of comb (hereafter, combs) but neonicotinoid residues with a one-third to one-sixth of an administration concentration could be detected from honey. It was assumed that the difference in residual pesticide between a neonicotinoid and an organophosphate would possibly come from the difference in the persistency of a pesticide, where a short-term persistent organophosphate would be easily decomposed during storage in cells but a long-persistent neonicotinoid, which was stored after having being diluted by pesticide-free nectar, would be very little decomposed during storage. It was concluded from these results that the concentrations of a pesticide in our previous experiments might not always be unrealistic in Japan.

A few researchers in Japan insisted that the chief cause of a massive colony loss was not a neonicotinoid pesticide but mites infesting honeybees against our findings that a neonicotinoid would be deeply involved in the massive colony loss judging from a fact that mites and wax worms (wax moths) cannot exist before colony extinction (Yamada et al., 2018c). Lu et al. (2014) also conducted the field experiment under more realistic concentrations of the neonicotinoids imidacloprid and clothianidin than their previous work (Lu et al., 2012), where the measurements such as the numbers of bees and brood by visual estimation where their validity cannot be checked again in the future analysis seem too rough in accuracy and insufficient and the experimental conditions such as a midstream change in pesticide concentration and new insecticidal administration to exterminate mites seem extremely irregular through their experiment period to analyse their experimental results in detail. Table 2 shows the field experiments conducted by Lu et al. (2012, 2014) and Yamada et al. (2012, 2018a, b, c).

To verify the validity of our findings, we made a plan for a long-term field experiment in Maui where there were no mites harmful to honeybees (*Apis mellifera*) and no seasonal changes without distinct winter. In this work, the field experiment was conducted in Maui under the same conditions as those of the experiment in Japan with exception of the differences in weather and pest (Yamada et al., 2018c).

MATERIALS AND METHODS

Materials and preparation of pesticide concentrations

STARCKLE MATE® (10% dinotefuran; Mitsui Chemicals Aglo, Inc., Japan), DANTOTSU® (16% clothianidin;

Table 1: Extracts from Maximum Residue Limits (MRLs) list of agricultural chemicals in foods in Japan (JFCRF, 2017).

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 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
Grape	5.00	5.00	15.00	3.00

Note: Uniform limit is 0.01 ppm.

Sumitomo Co. Ltd., Tokyo, Japan) and SUMITHION® emulsion (50% fenitrothion; Sumitomo Co. Ltd;, Japan) were used after being dissolved in sugar syrup was made by mixing cane sugar purchased from Maui COSTCO Warehouse with the same weight of water in this study. On comparing the effects of these pesticides on a honeybee (*A. mellifera*) colony, we prepared one-five hundredth of each concentration of pesticides recommended by their manufacturers to exterminate stinkbugs which are representative insect pests in Japan. Each concentration of pesticides used in this study was 0.2 ppm for dinotefuran, 0.08 ppm for clothianidin and 1 ppm for fenitrothion which are much lower than a concentration detected near rice paddy fields in Japan such as about 5 ppm of clothianidin near a rice field reported by Kakuta et al. (2011) and about 0.7 ppm of dinotefuran in the run-off from paddy fields reported by Yokoyama et al. (2015). It was confirmed that the concentrations of dinotefuran and clothianidin which are prepared to have the same insecticidal activity affecting stinkbug have almost the same effect on honeybees as reported by Yamada et al. (2012).

Methods used in field experiments

Experiments were conducted from October 22nd 2014 to July 20th 2015 in the flat land (Latitude 20º55'00.2" N, Longitude 156º30'39.2" W) which is 220 acres of organically-grown macadamia trees in Wailuku in west Maui (1070 to 1090, Malaihi Rd., Wailuku, HI, USA) surrounded by the sea and non-commercial organically-grown farmlands around which there is a vast nature conservation area. Honeybees can take their foods (nectar and pollen) at any time of the year in the experimental site in Maui. For example, a macadamia tree comes into flower six times in a year. The stage of the bloom ranges from

Table 2: Comparison of the influence of a pesticide on a honeybee colony by long-term field experiments among recent articles.

Variable	**(Lu et al., 2012)**	**(Lu et al., 2014)**	**(Yamada et al., 2012)**	**(Yamada et al., 2018a)**	**(Yamada et al., 2018b)**	**(Yamada et al., 2018c)**
Title of article	In situ replication of honeybee colony collapse disorder	Sublethal exposure to neonicotinoids impaired honey bees winterization before proceeding to colony collapse disorder	Influence of dinotefuran and clothianidin on a bee colony	A clear difference in the impact on a honeybee Apis mellifera colony between the two vehicles of sugar syrup and pollen paste through which dinotefuran is administered in a long-term field experiment	An evident difference between the impact of the neonicotinoid dinotefuran and that of the organophosphate fenitrothion on a bee colony in a long-term field experiment on the assumption of their comparatively high concentrations around crop-dusted areas	Comparison of the influence of a pesticide at an environmentally realistic concentration level in Japan on a honeybee colony between neonicotinoids (dinotefuran, clothianidin) and organophosphates (fenitrothion, malathion)
Experimental period	July 22nd 2010 to September 30th (70 days)	October 27th 2012 to April 4th 2013	July 18th 2010 to November 21st (126 days)	July 9th 2011 to April 2nd 2012 (268 days)	June 28th 2012 to July 26th 2013 (393 days)	August 13th 2013 to February 28th 2014 (199 days)
Object of Study	Influence of high-fructose corn syrup (HFCS) made of maize produced by imidacloprid-treated seeds on honeybees	To extend their previous study (Lu et al., 2012) showing that sublethal exposure of imidacloprid and clothianidin affected the winterization of healthy honey bee colonies that subsequently leads to a colony collapse disorder (CCD)	To investigate the possibility for a neonicotinoid of causing a CCD	To investigate the difference in the long-term influence on a bee colony between toxic sugar syrup (honey) as an energy source and toxic pollen paste (bee bread) as a protein source which are exposed to a neonicotinoid	To investigate the difference in the long-term influence on a bee colony between a neonicotinoid and a organophosphate which are administered through sugar syrup	To investigate the difference in the long-term influence on a bee colony between a neonicotinoid and a organophosphate which are administered through sugar syrup under lower concentrations than in the previous work
Circumstances around Experimental Site						
Limitation of honeybee activities	Honeybees can freely forage about for food in a hive or in fields	Honeybees can freely forage about for food in a hive or in fields	Honeybees can freely forage about for food in a hive or in fields	Honeybees can freely forage about for food in a hive or in fields	Honeybees can freely forage about for food in a hive or in fields	Honeybees can freely forage about for food in a hive or in fields
Impact of other pesticide than the administered one on the environment	Unknown	Unknown	A pesticide-free watering place & a pesticide-free field of flowers in the apiary	A pesticide-free watering place & a pesticide-free field of flowers in the apiary	A pesticide-free watering place & a pesticide-free field of flowers in the apiary	A pesticide-free watering place & a pesticide-free field of flowers in the apiary
Aerial-crop-dusting farmland near exptl. Site	Unknown	Unknown	Nothing	Nothing	Nothing	Nothing
Seasonal changes in weather	Distinct changes in weather among four seasons	Distinct changes in weather among four seasons	Distinct changes in weather among four seasons	Distinct changes in weather among four seasons	Distinct changes in weather among four seasons	Distinct changes in weather among four seasons
Location of experimental site	Southern Worcester County located in Central Massachusetts, USA. Sites were located at least 12 km away from each other	Unknown	Latitude 20º55’00.2” N, Longitude 156º30’39.2” W	Latitude 20º55’00.2” N, Longitude 156º30’39.2” W	Latitude 20º55’00.2” N, Longitude 156º30’39.2” W	Latitude 20º55’00.2” N, Longitude 156º30’39.2” W
Experimental conditions						
Initial numbers of apiaries, colonies and combs (frames)	4 apiaries, 5 colonies per apiary, 20 combs per colony	3 apiaries, 6 colonies per apiary, 18 combs per colony	1 apiary (private), 8 colonies per apiary, 6 combs per colony	1 apiary (private), 5 colonies per apiary, 3 combs per colony	1 apiary (private), 4 colonies per apiary, 3 combs per colony	1 apiary (private), 6 colonies per apiary, 3 combs per colony

Table 2: Conts. Comparison of the influence of a pesticide on a honeybee colony by long-term field experiments among recent articles.

Initial number of bees per colony	adult bees of 1.4 kg per hive	5000	9000 to 1300 bees (accurately counted on photos)	1700 to 3400 bees (accurately counted from photos)	5600 to 7100 bees (accurately counted on photos)	5400 to 7600 bees (accurately counted on photos)
Initial number of capped brood per colony	Contained at least 14 frames of capped brood	Unknown	1.5 to 7 full-surfaces on comb converted from the sum total of every area occupied by capped (obtained from photos)	2600 to 6100 capped brood (accurately counted from photos)	4000 to 5700 capped brood (accurately counted from photos)	4200 to 7600 capped brood (accurately counted from photos)
Kind of pesticide	Imidacloprid	Imidacloprid, Clothianidin	Dinotefuran, Clothianidin	Dinotefuran	Dinotefuran, Fenitrothion	Dinotefuran, Clothianidin, fenitrothion, Malathion
Concentration of pesticide		110.6 ppb 1000 * 258 μg /(0.5 *gal* * 3.7853 *l*/*gal* * 1232 *g* /L)	Dinotefuran: 1, 2, 10 ppm, Clothianidin: 0.4, 2, 4 ppm	Dinotefuran: 1 and 10 ppm in sugar syrup, 0.565 & 5.65 in pollen paste	Dinotefuran: 2 ppm in sugar syrup, Fenitrothion: 10 ppm in sugar syrup	Dinotefuran: 0.2 ppm in sugar syrup, Clothianidin: 0.08 ppm in sugar syrup, Fenitrothion and Malathion: 1 ppm in sugar syrup
Origin of a queen	Unknown in detail (*Apis mellifera*) Bee colonies purchased from a bee farm	Unknown in detail (*Apis mellifera*) Bee colonies purchased from a bee farm	Unknown in detail (*Apis mellifera*) Bee colonies purchased from a bee farm	Unknown in detail (*Apis mellifera*) Bee colonies purchased from a bee farm	Unknown in detail (*Apis mellifera*) Bee colonies purchased from a bee farm	Unknown in detail (Apis mellifera) Bee colonies purchased from a bee farm
Kind of syrup	HFCS; From November in 2012, either with crystallized HFCS or with granular sucrose mixed i	HFCS; From November in 2012, either with crystallized HFCS or with granular sucrose mixed into a thick water paste	Sugar syrup purchased from The Japan Beekeeping Association	Sugar syrup purchased from The Japan Bee-keeping Association	Sugar syrup purchased from The Japan Beekeeping Association	Sugar syrup purchased from The Japan Beekeeping Association
Kind of pollen			Pure pollen made in Spain which was purchased from a bee-keeper in Japan	Pure pollen made in Spain which was purchased from a bee-keeper in Japan	Pure pollen made in Spain which was purchased from a bee-keeper in Japan	Pure pollen made in Spain which was purchased from a bee-keeper in Japan
Experimental methods						
Interval of experiment	About two weeks-interval for each apiary	One weeks-interval for each apiary	About one-week interval	About one-week interval	About one-week or two-weeks interval	About one-week or two-weeks interval
Confirmation and record methods of a queen	Unknown	Unknown	A photographic record of the existence of a queen in each colony	A photographic record of the existence of a queen in each colony	A photographic record of the existence of a queen in each colony	A photographic record of the existence of a queen in each colony
Administration period of pesticide	July 1st 2010 to September 30th (91 days)	July 2nd 2012 to September 17th 2012 (77 days)	July 18th 2010 to November 21st (126 days)	July 9th 2011 to December 3rd (147 days)	July 21st 2012 to August 16th (26 days)	September 5th in 2013 to December 1st (87 days)
Form in performing an experiment	Unknown (we can guess that authors might made a experimental plan and bee-keepers might carried out it)	Unknown (we can guess that authors might made a experimental plan & bee-keepers might carried out it)	Authors made an experimental plan and carried out it	Authors made an experimental plan and carried out it	Authors made an experimental plan and carried out it	Authors made an experimental plan and carried out it
Starting time of each observation	Unknown	Unknown	Just after dawn if possible (before bees go out there foraging)	Just after dawn if possible (before bees go out there foraging)	Just after dawn if possible (before bees go out to there foraging)	Just after dawn if possible (before bees go out to ther foraging)
Making and feeding methods of sugar syrup		Either with crystallized HFCS or with granular sucrose mixed into a thick water paste	Sugar syrup was made after having dissolved sugar in the same weight of water as it	Sugar syrup was made after having dissolved sugar in the same weight of water as it	Sugar syrup was made after having dissolved sugar in the same weight of water as it	Sugar syrup was made after having dissolved sugar in the same weight of water as it

Table 2: Conts. Comparison of the influence of a pesticide on a honeybee colony by long-term field experiments among recent articles.

Making and feeding methods of pollen paste	Unknown	Unknown	Pollen paste with a moderate viscosity was made after pollen was mixed with sugar syrup and was kneaded	Pollen paste with a moderate viscosity was made after pollen was mixed with sugar syrup and was kneaded	Pollen paste with a moderate viscosity was made after pollen was mixed with sugar syrup and was kneaded	Pollen paste with a moderate viscosity was made after pollen was mixed with sugar syrup and was kneaded
Administration method of pesticide	Dissolved in HFCS. Each apiary consisted of 4 different imidacloprid-treated hives and a control hive. Imidacloprid was initially fed to honey bees at 0.1, 1.1, 5.3, and 10.5 μg/kg in HFCS per week for 4 weeks starting from July 1st 2010, followed by 20, 40, 200, and 400 μg/kg per week for additional 9 weeks, which ended on September 30th 2010.	Starting from November in 2012, hives were supplemented either with crystallized HFCS or with granular sucrose mixed into a thick water paste	A pesticide was dissolved in sugar syrup and pollen was kneaded with toxic sugar syrup containing the pesticide. Both toxic sugar syrup and toxic pollen paste were fed into a hive.	A pesticide was dissolved in sugar syrup or pollen was kneaded with toxic sugar syrup containing the pesticide. Either toxic sugar syrup or toxic pollen paste was fed into a hive.	A pesticide was dissolved in sugar syrup or pollen was kneaded with toxic sugar syrup containing the pesticide. Only toxic sugar syrup was fed into a hive.	A pesticide was dissolved in sugar syrup or pollen was kneaded with toxic sugar syrup containing the pesticide. Only toxic sugar syrup was fed into a hive.
Vehicle to administer a pesticide	Syrup (high fructose corn syrup (HFCS)	Syrup (sucrose water or high fructose corn syrup (HFCS))	Both sugar syrup and pollen paste	Sugar Syrup or pollen paste	Sugar syrup	Sugar syrup
Criteria to determine a concentration of pesticide	Ten times the concentration of the tolerance set by the US Environmental Protection Agency (EPA)		Consulting the concentration of clothianidin in water near a paddy field & the maximum residue limits (MRLs) in food in Japan	Consulting the concentration of clothianidin in water near a paddy field & the maximum residue limits (MRLs) in food in Japan	Consulting the concentration of clothianidin in water near a paddy field & the maximum residue limits (MRLs) in food in Japan	Consulting the concentration of clothianidin in water near a paddy field
Counting method of the number of adult bees (worker bees)	During this scoring process notes were also made of the number of frames of adult bees observed.	Round number from combs with bees by observation within 10 s. Notes were taken on the size of the clusters observed by counting the numbers of frames containing honey bees from the top of the hive in which it generally took no more than 10 s.	Accurately counted from photos of combs with bees and bees left in a hive after every comb was removed from it	Accurately counted from photos of combs with bees and bees left in a hive after every comb was removed from it	Accurately counted from photos of combs with bees and bees left in a hive after every comb was removed from it	Accurately counted from photos of combs with bees and bees left in a hive after every comb was removed from it
Counting method of the number of capped brood	Obtained by A modified brood assessment method (Scored by visually estimating the number of squares of capped brood per frame	Obtained by A modified brood assessment method (Scored by visually estimating the number of squares of capped brood per frame	Expressed by the number of surfaces on combs (frames) which are entirely occupied by capped brood using the photos of combs	Accurately counted from photos of combs without bees after shaking the bees off each comb	Accurately counted from photos of combs without bees after shaking the bees off each comb	Accurately counted from photos of combs without bees after shaking the bees off each comb

Table 2: Conts. Comparison of the influence of a pesticide on a honeybee colony by long-term field experiments among recent articles.

Total intake of pesticide per colony	Un-used syrup with ididacloprid was measured and discarded and exposure calculations adjusted accordingly, although the incomplete		Calculated from the sugar syrup & pollen paste with pesticide consumed by honey bees	Calculated from the sugar syrup & pollen paste with pesticide consumed by honey bees	Calculated from the sugar syrup & pollen paste with pesticide consumed by honey bees	Calculated from the sugar syrup & pollen paste with pesticide consumed by honey bees
Estimation of the intake of pesticide per bee	Unestimated	Assuming each colony consisted of 50,000 bees at any given day in spring and summer, we administered 0.74 ng/bee/day of either imidacloprid or clo•thianidin to treated hives for 13 consecutive weeks.	Unestimated	Estimated from dividing the total intake of pesticide per colony by the total number of initial and newly-emerged honeybees	Estimated from dividing the total intake of pesticide per colony by the total number of initial and newly-emerged honeybees	Estimated from dividing the total intake of pesticide per colony by the total number of initial and newly-emerged honeybees
Counting method of number of dead bees	Uncounted	Number of dead bees existing on the bottom	Dead bees were accurately counted one by one inside and outside a hive which was placed on a large tray	Dead bees were accurately counted one by one inside and outside a hive which was placed on a large tray	Dead bees were accurately counted one by one inside and outside a hive which was placed on a large tray	Dead bees were accurately counted one by one inside and outside a hive which was placed on a large tray
Main findings	1) 15 of 16 imidacloprid treated hives (94%) were dead across 4 apiaries 23 weeks post imidacloprid dosing. Dead hives were remarkably empty except for stores of food and some pollen left, a resemblance of CCD.	1) Sublethal exposure of neonicotinoids, imidacloprid or clothianidin, affected the winterization of healthy colonies that subsequently leads to CCD. Bees in both control and neonicotinoid treated groups progressed almost identically through the summer and fall seasons and observed no acute morbidity or mortality in either group until the end of winter. Bees from six of the twelve neonicotinoid treated colonies had abandoned their hives, and were eventually dead with symptoms resembling CCD.	1) Each experimental colony where a neonicotinoid pesticide (dinotefuran, clothianidin) was administered became extinct just after having assumed a CCD phenomenon aspect.	1) An experimental colony where dinotefuran was administered through pollen paste became extinct at only about one-fifth of the intake per bee of dinotefuran where was administered through sugar syrup.	1) An impact of the organophosphate fenitrothion on a bee colony was quite different from that of the neonicotinoid dinotefuran. Dinotefuran colony became extinct as in the case of our previous works, however, fenitrothion colony did not become extinct, but also even succeeded in overwintering as in the case of a control colony.	1) A neonicotinoid colony where either dinotefuran or clothianidin was administered through sugar syrup became extinct much earlier than an organophosphate colony where either fenitrothion or malathion was administered through sugar syrup under lower concentrations of pesticide as in the case of our previous results.

Table 2: Conts. Comparison of the influence of a pesticide on a honeybee colony by long-term field experiments among recent articles.

	2) Control hives survived after winter.	2) A complete opposite phenomenon in the control colonies in which instead of abandonment, they were repopulated quickly with new emerging bees. Only one of the six control colonies was lost due to Nosema-like infection.	2) Every experimental colony definitely decreased in both the numbers of adult bees and brood while a difference in numbers between experimental and control colonies were widening and finally became extinct.	2) Every experimental colony must have become extinct, while assuming a CCD aspect as in the case of our previous work except the colony where 10 ppm dinotefuran was administered.	2) Dinotefuran colony became extinct only for 26 days during the administration of dinotefuran in this work, though it became extinct for 61 days under the same concentration of dinotefuran in our previous work (Yamada et al., 2012). It was deduced that the difference in survival period was due to the difference in weather.	2) An organophosphate colony became extinct late in overwintering. It was deduced that the reason why the colony extinction might be caused by the turbulence of a bee cluster due to the experiment in a cold day early in January. Judging from the extinction of a control colony during overwintering, it was assumed that a difference in colony activity between a control colony and a organophosphate colony would be small though a control colony might be slightly more active than a organophosphate colony.
Prevention of infectious disease	Before wintering, all hives were treated with Apis tan strips and Fumagilin B for parasite prevention	Treated with Mite away Quick strips for controlling Varroa mite on August 13th in 2012, followed by Apis tan strips from October 1st to November 15th 2012. October, 2012 to control *N. apis* and *N. ceranae*, two common intestinal parasites	No treatment	No treatment	No treatment	No treatment

January to June. Twelve beehives, each with three combs and a feeder to feed sugar syrup were sited in four lines (same pesticide) and three rows (different pesticides) facing south as shown in Figure 1. In order to prevent various errors such as mistakes in taking pictures and counting the numbers of adult bees and brood, the frame of comb was marked with a number and symbols to express both sides of a comb and a kind of a pesticide as in the case of our previous experiments (Yamada et al., 2012, 2018a, b, c). A comb was numbered on the frame in order from the right toward the entrance of a hive. Both surfaces of a comb were marked with the symbol "F" to the right towards the entrance of a hive and "B" to the left, respectively. Each colony was expressed by abbreviating a control colony to "CR1, CR2 and CR3"; a dinotefuran colony where dinotefuran was administered to "DF1, DF2 and DF3"; a clothianidin colony where clothianidin was administered to "CN1, CN2 and CN3"; a fenitrothion colony where fenitrothion was administered to "FT1, FT2 and FT3". For example, DF2-3B means the left surface of third comb towards the entrance of a hive in the dinotefuran colony on the second row. Each hive is installed an auto-feeding system from which sugar syrup (weight ratio of cane sugar is equal to that of water) can be automatically and continuously fed to the small tray placed on the bottom of the hive through a 10 L (about 14 kg of sugar syrup) container as in the case of Yamada et al. (2018c). Figure 2 shows the photograph of an auto-feeder system. 12 kg of sugar syrup was fed to each colony. When the container containing a pesticide dissolved in became empty, sugar syrup containing the pesticide was newly filled up after the weight of sugar syrup remaining was accurately measured and the container washed. The consumption of sugar syrup was calculated. 12 kg of pesticide-free sugar syrup was fed only once into each control colony on October 23rd 2014. Given the fact that honeybees can get nectar comparatively easily in Maui, we assumed that

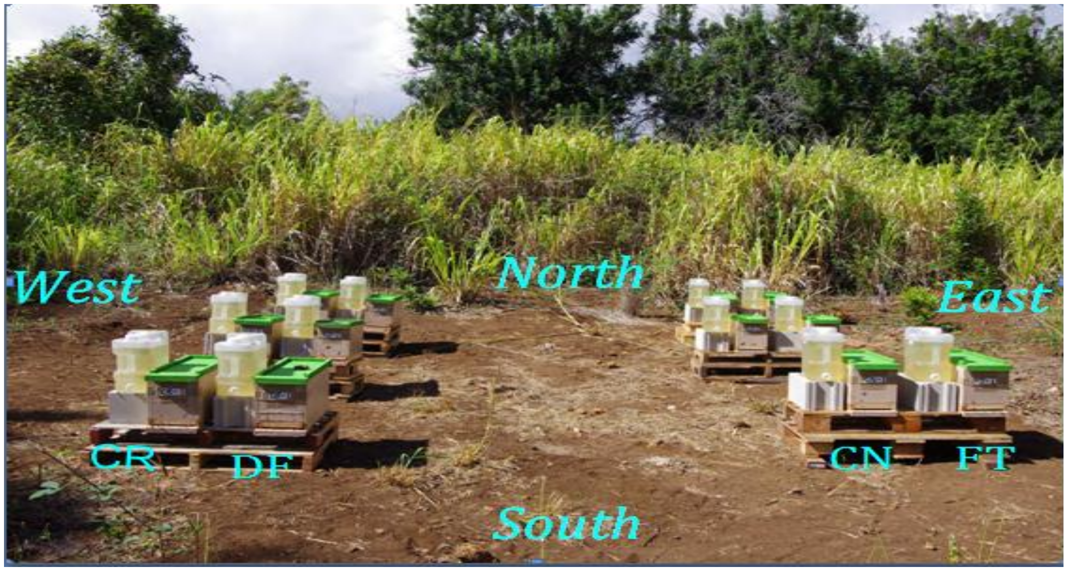

Figure 1: Arrangement of hives in the experimental site. Twelve beehives were sited in four lines (same pesticide) and three rows (different pesticides) facing south.

Figure 2: Auto-feeding system of sugar syrup. Sugar syrup stored in 10 L-container is automatically and continuously fed to the auto-feeder on the bottom of the beehive through the flexible tube as much as honeybees have taken.

whether sugar syrup was fed into a control colony or not seemed to slightly affect honeybees on nutrition.

The experiment was started from October 22nd 2014 under the condition that pesticide-free sugar syrup was fed to every colony. On the next day, October 23rd 2014, sugar syrup containing a pesticide was administered into every experimental colony. We decided on finishing the experiment when every colony has become extinct or at the end of the season (the end of July in 2015) when there are a few flowers in bloom after passing the stage of full bloom because toxic honey containing a pesticide which is stored in cells of combs may probably be ingested by honeybees – whichever comes first. Each pesticide decided on finishing the administration at the end of March or at the beginning of April just before flowers will be in full bloom because honeybees prefer to ingest nectar rather than sugar syrup and store sugar syrup in cells in the stage of full bloom or when every colony has become extinct – whichever comes first.

All queens used in this experiment are sisters which

Table 3: Queen assessment sheet by Maui Queen Co.

Grafting date	Breeder colony	Grafter	No. of grafts	Cell builder yard	Cloak board colony	No. of grafts accepted	Mating yard (Date moved)	Breeder Queen description	
17-8-14	V.S.H. Blue 5	Paul Apao assisted by Lloyd Fischel	60	1	2	54	1 (27-Aug-14)	Color	Yellow/Tiger
								Race	Italian/German
								Size	Large
								Worker color	Medium dark
								Behavior	8 (1: the most aggressive, 10: the least aggressive)
								Laying	Good pattern
								Honey	Good production
								Hygienic	8 (1: the least hygienic, 10: the most hygienic)
								Drones	Yes

emerged at the same time (referred to Queen Assessment Sheet shown in Table 3). We endeavoured to start the experiment (taking photographs) just after dawn (at about half past six) when few bees go foraging about for food. Some bees, however, may have gone foraging at the end of the experiment because it takes about three hours or more to conduct the experiment. When the heavy downpour makes continuance of the experiment impossible, the experiment will be suspended temporarily at a good point to leave off and thereafter the experiment will be resumed as soon as possible after the weather gets better. If the experiment cannot be resumed in the course of the day, the experiment will resume another day weather permitting.

We endeavoured to record the observational results in photographs as much as possible. The typical experimental procedures are as follows: 1) We take a few photographs of an overall view of the experimental site; 2) we count the dead bees in the large tray on which a hive is placed one after the other while picking up with a pair of tweezers. If there are many dead bees, we count them after the end of the experiment; 3) after we take a photograph of the front of a hive, we take photographs of the inside of the hive. That is to say, after we open the top cover of the hive, we take a photograph of the top of the hive. Thereafter, we take photographs of both surfaces of each comb on which adult bees exist while drawing out each comb in ascending numeric order of comb number (in order from the right towards the entrance of the hive) and thereafter, putting it in another empty hive. After we have taken the photographs of both surfaces of every comb, we take photographs of four walls and the bottom of the hive inside where there are adult bees left after we have drawn out every comb. After taking photographs of four walls and the bottom, we count dead bees while taking dead bees out of the box with a pair of tweezers. If the inside of the hive is dirty, we make clean or exchange the hive for another clean hive. When taking photographs, we confirmed the existence of a queen. When we have found the queen, we take a few photographs of the queen while enlarging it and thereafter isolated the queen in a queen cage during taking photographs. When we cannot find the queen during the drawing-out of every comb, we carefully examine the inside of the hive severally and every comb drawn out. However hard we may try we cannot find the queen, we suppose that there may be no queen in the colony and we will look for the queen in the next experiment. If we cannot find out the queen next time anyhow, we conclude that there is no queen in the colony. After we have taken photographs of all the adult bees, we take photographs of capped brood as follows. We draw out the comb with bees stored in another hive in ascending numeric order of comb number and then we take photographs of both surfaces of the comb without bees after shaking all the bees off the comb into the original hive. After taking photographs of the comb without bees, we return the comb without bees at the original position in the original hive in ascending numeric order of comb number. After taking photographs of all the comb and returning them into the hive, we carefully put the queen in a queen cage into the original hive. When we find out

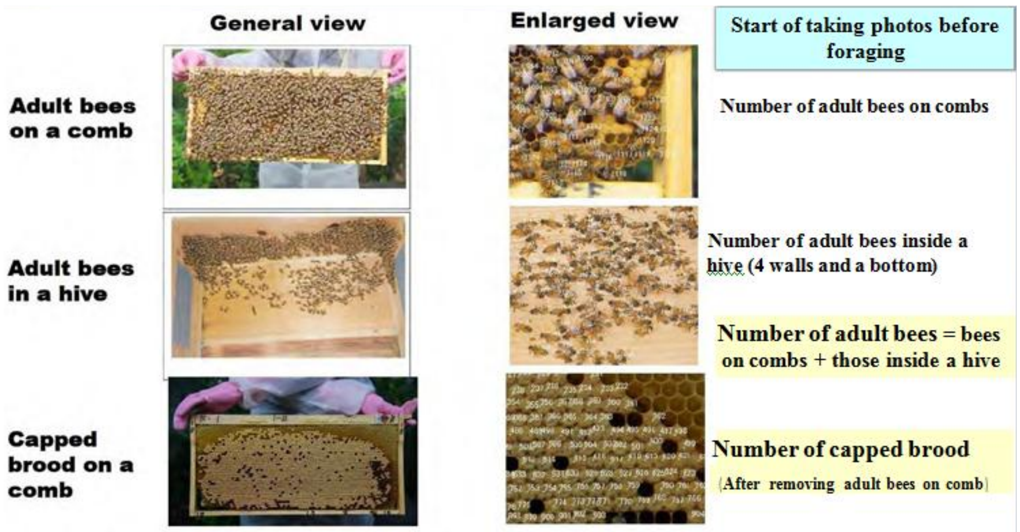

Figure 3: Examples of measuring numbers of adult bees and capped brood. Development of image processing software ➡Counting them in round numbers ➡ Correction of the round numbers while being visually and carefully examined after enlarged.

any signs of irregularity such as small hive beetles, wax moth larvae, queen cells, some diseases (e.g., chalk brood) and mites (mites cannot exist in Maui), we take photographs of the sign while sometimes enlarging it. When adult bees are unusually abundant in a hive, we add one or two new combs into the hive. We check the residual quantity of sugar syrup in the 10-L container. We write the details involving items warranting special mention observed during the experiment in the data sheet for observational results. After the experiment, we check all of the photographs on a camera. If there are any problems, we begin again on the problems as soon as possible. Where the temperature, humidity and dew point values were continuously recorded on a data logger (EL-USB-2; Lascar Electronics Inc.) in the hives CR-2, DF-2, CN-2 and FT-2 and out the hives of CR-1 (at the corner of the south-west of the experimental site) and FT-3 (at the corner of the north-east of the site).

Thereafter, we carefully check all the photographs on a computer display. If we find any important problem, we start all over from the beginning. When we cannot find any problem, we write captions for all the photographs to prevent mistakes when counting the numbers of adult bees, brood and mites, grasping the situation of the experiment, reaffirming and analysing the observational results. Such a photographically recording method will enable a review or a re-measurement of these data afterwards and a future analysis of new data such as the numbers of mites and wax-worm larvae through the photographs.

Counting methods of adult bees, brood, mites and dead bees

Figure 3 shows the numbers of adult bees, brood and mites counted on a photograph taken in the experiment. The total number of adult bees on every comb which was numbered and ordered numerically in a beehive, and those on the inside of the beehive box (4 walls and bottom) was counted directly and accurately on photographs (sometimes enlarged) of all combs while correcting carefully the number of adult bees estimated by improved "Perfect Viewer 7" developed by Nanosystem Corporation, Japan. The total number of capped brood was counted in a similar manner, after directly shaking the bees off each comb as shown in Figure 3. Though we tried to develop a new automatic counting software with binarizing photograph images (improved "Perfect Viewer 7") which was jointly developed with Nanosystem Corporation, we cannot always succeed in highly accurate counting of them (with an error of not over ± 3%) because it cannot accurately count overlaid bees, bees and capped brood on blurred image, those on low contrast or low brightness even when changing the threshold. We checked the accuracy of the measurement by comparing the number of adult bees on the same photograph counted among three persons and three repeats by three same individuals with a margin of error among all the measurements was within 3%, where we selected several photographs as samples to check the accuracy of measurement. We selected the following samples for cross-checking with three persons: A few photographs with more than 1200 of adult bees partially overlapping on the surface of comb, those with more than 400 of adult bees on a wall or the bottom of the hive and those with more than 1500 of capped brood on the surface of comb with some capped honey. In the case of the automatic counting software, a margin of error in the number of adult bees between software counting and human counting with the help of the software counting was approximately 20 to 30%.

Table 4: State of colony in 2014 Maui experiment.

Date	State of colony as of July 20th 2015, active or extinct											
	Control			Dinotefuran			Clothianidin			Fenitrothion		
	CR-1	**CR-2**	**CR-3**	**DF-1**	**DF-2**	**DF-3**	**CN-1**	**CN-2**	**CN-3**	**FT-1**	**FT-2**	**FT-3**
22-Oct-14	Active	Active	Active	Active	Active	Active	Active	Active	Active	Active	Active	Active
23-Oct-14	Active	Active	Active	Active	Active	Active	Active	Active	Active	Active	Active	Active
24-Oct-14	Active	Active	Active	Active	Active	Active	Active	Active	Active	Active	Active	Active
25-Oct-14	Active	Active	Active	Active	Active	Active	Active	Active	Active	Active	Active	Active
28-Oct-14	Active	Active	Active	Active	Active	Active	Active	Active	Active	Active	Active	Active
30-Oct-14	Active	Active	Active	Active	Active	Active	Active	Active	Active	Active	Active	Active
20-Nov-14	Active	Active	Active	Active	Active	Active	Active	Active	Active	Active	Active	Active
10-Dec-14	Active	Active	Active	Active	Active	Active	Active	Active	Active	Active	Active	Active
29-Dec-14	Active	Active	Active	Active	Active	Active	Active	Active	Active	Active	Active	Active
17-Jan-15	Active	Active	Active	Active	Active	Active	Active	Active	Active	Active	Active	Active
30-Jan-15	Active	Active	Active	Active	Active	Active	Active	Active	Active	Active	Active	Active
18-Feb-15	Active	Active	Active	extinct	Active	Active	Active	Active	Active	Active	Active	Active
17-Mar-15	Active	Active	Active	extinct	Active	Active	Active	Active	Extinct	Active	Active	Active
4-Apr-15	Active	Active	Active	extinct	extinct	Active	Active	Active	Extinct	Extinct	Active	Active
11-May-15	Active	extinct	Active	extinct	extinct	Active	Active	Active	Extinct	extinct	Active	Active
2-Jun-15	Active	extinct	Active	extinct	extinct	Active	Active	Extinct	Extinct	extinct	Active	Active
20-Jul-15	Active	Extinct	Active	Extinct	Extinct	Extinct	Extinct	Extinct	Extinct	Extinct	Active	Active
15-Oct-15	Active	Extinct	Extinct after swarming	Extinct	Extinct	Extinct	Extinct	Extinct	Extinct	Extinct	Active	Active
5-Nov-15	Active	Extinct	Extinct	Extinct	Extinct	Extinct	Extinct	Extinct	Extinct	Extinct	Queenless (almost extinct)	Active

On July 20th, the colonies of CR-1, CR-3, FT-2 and FT-3 were active and the colonies of CR-2, DF-1, DF-3, CN-1, CN-2, CN-3 and FT-1 became extinct. The extinction rate of a colony as of July 20th 2015 is 33.33% for control (pesticide free) colonies and organophosphate (fenitrothion) colonies. It is 100% for neonicotinoid (dinotefuran and clothianidin) colonies. That is, the survival ratio of a colony is 66.67 % for control and organophosphate colonies and 0% for neonicotinoid colonies.

On the other hand, a margin of error in the number of capped brood is approximately 10 to 20% between them. The automatic counting software can give a better estimation of the number of capped brood than that of the number of adult bees. The errors involved in estimation of the number of adult bees by the automatic counting software seem to be mainly ascribed to such causes as the overlapping of bees, a blurred image due to moving bees and an insufficiency of light quantity, a bee getting into a cell and unevenness in brightness on an image due to shadows. On the other hand, the errors in measurements of the number of capped brood seem to be mainly ascribed to such causes as a misreading of a capped honey cell as a capped brood cell and an imperfectly capped brood.

We could not find mites in every colony, though we carefully examined every photograph enlarged on a display of the computer. The total number of dead bees in the tray, feeder and hive was counted directly, one after the other with a pair of tweezers because it is very difficult to judge whether a bee is dead or alive on a photograph with many bees.

RESULTS

Outline of long-term observations

The field experiment was conducted in Maui from October 22nd 2014 to July 20th 2015 (271 days). Table 4 shows the state of colony of whether a colony is active or not in each observational date over the experimental period. All of the neonicotinoid colonies into which a neonicotinoid pesticide (dinotefuran or clothianidin) was administered became extinct during the experimental period. On the other hand, only one of three fenitrothion colonies where the organophosphate pesticide fenitrothion was administered became extinct during the experimental period as in the case of three control colonies where no pesticide was administered. That is to say, the survival rate of neonicotinoid (dinotefuran and clothianidin) colony is zero percent and those of organophosphate (fenitrothion) colony and control colony are about 67%. These results support our previous findings that the survival rate of neonicotinoid colony was 0% and that of organophosphate colony was approximately equal to that of control colony in Japan.

Table 5 shows an outline of experimental observations picked out from our research note and a data sheet on each observation date. In Table 5 only visually observed results (October 15th 2015 and November 5th) are added after the end of the experiment on July 20th where the points of observations without taking photographs were jotted down. Here, we will explain briefly the general outlines on the situation of the colonies which became extinct in this work based on the state of colony (Table 4) and the remarks shown in Table 5. Twelve honeybee colonies were prepared for this experiment and divided into four groups composed of three control colonies, three dinotefuran colonies, three clothianidin colonies and three fenitrothion colonies. Eight colonies became extinct during the experimental period, in which six colonies were neonicotinoid colonies (DF-1, DF-2, DF-3; CN-1, CN-2 and CN-3), one colony was a control colony (CR-3) and the other colony was an organophosphate colony (FT-1). The neonicotinoid DF-1 became extinct first on February 18th 2015 (118 days elapsed from the start of pesticide administration). Additionally, the neonicotinoid CN-3 became extinct secondly on March 17th 2015 (145 days). Thereafter, both the neonicotinoid DF-2 and the organophosphate FT-1 became extinct on April 4th 2015 (174 days). Subsequently, the pesticide-free CR-2 became almost extinct on May 11th 2015 (200 days), where there were no queen from April 4th 2015; only about five hundred adult bees and three capped brood in the hive, and accordingly, we accepted it as colony extinction. Thereafter, the neonicotinoid CN-2 became extinct on June 2nd 2015 (222 days). It was visually confirmed that the neonicotinoid DF-3 and CN-1 had already become extinct on July 20th 2015 after a long absence from the experimental site, where we regrettably forgot to take photographs but committed the fact to writing in our research note on that day. Regarding the conditions in the extinct colony, we found a difference between the neonicotinoid colonies and the other colonies as follows: There were no wax-moth larvae in every one of the six neonicotinoid colonies (DF-1, DF-2, DF-3, CN-1, CN-2 and CN-3) but there were several wax-moth larvae in the control CR-2 and the organophosphate FT-1 when they became extinct.

Table 5 also showed the detailed conditions in each colony, in which are tabulated an experimental date and elapsed days from the start of experiment which is one-day longer than elapsed days from the start of pesticide administration, the starting time of experiment in an experimental date, the temperature at the experimental site at the start-up time, kind of pesticide, the point of the queen in the hive, the existence of queen cells and the number of frames with comb.

Number of dead bees

The number of dead bees in a colony was obtained by summing up the number of dead bees on the large tray laid under the hive and that inside the hive such as on the bottom, in a feeder of sugar syrup laid on the bottom and sometimes in cells of combs, where all dead bees were tossed away out of the hive one after the other while being counted to avoid counting dead bees in mistake for adult bees on photographs. Table 6 shows the number of dead bees in a colony obtained from such procedure. It can be seen from Table 6 that there were a few dead bees in each colony, at the most, 125 over the experimental period.

Number of adult bees

The number of adult bees in a colony was obtained by summing up the number of adult bees on every comb and that inside the hive such as on four walls and the bottom, which was shown in Table 7 and illustrated in Figure 4. Figure 4 demonstrates that roughly speaking, every colony analogously changes with the seasons except when a colony has become extinct. At present, we examined the changes in the number of adult bees. Adult bees gradually decreased in number from late October, 2014 (the start of experiment) to late January, 2015, and then reached an approximate plateau in the number in the lowest level to late February and after that they rapidly increased in the number to late May. This change in the number of adult bees seems to correspond to change in weather, where January and June are the rainy season when there are few flowers in bloom in Maui and April and September are the dry seasons when there are many flowers in full bloom. The season when adult bees are sharply increasing as shown in Figure 4 coincides nearly with the season when many flowers including macadamia flowers are in full bloom around the experimental site, where a twelve-month change in the number of adult bees is estimated around the experimental site by Apao (2014), who is a bee-keeper in Maui based on his experience as shown in Figure 5.

Currently, we examined the change in the number of adult bees for each colony group in few details. Only one (CR-1) of three control colonies (CR-1, CR-2 and CR-3) shows similar change in the number of adult bees to the general trend of the twelve-month change, All conrol colonies show the similar trend in the number of adult bees till the end of January. But after late February, CR-2 turns to a very slight increase in the number of adult bees and

Table 5: Remarks in experimental observation.

Date	Remarks
2014/10/22 Start of experiment	1) We started to take photos of all combs and the inside of each beehive to count the numbers of adult bees and capped brood; 2) A sister queen was put in each beehive; 3) The queen in FT-3 was in a cage.
2014/10/23. Start of administration	1) We started to administer a pesticide into every experimental colony; 2) We could not find a queen in CN-1. We intended check it again the next day.
2014/10/24	1) Five queen cells could be found in FT-3; 2) The queen in the cage of FT-3 was released from the cage in the beehive of FT-3 after removing the five queen cells; 3) We found the queen in CN-1; 4) We could find a queen in every colony.
2014/10/25	1) DF-1 was low populations and had some hive beetles; 2) We could not find a queen in DF-2; 3) We could not find a queen in FT-3; 4) As we were apprehensive for the queenlessness of DF-2 and FT-3, we intended check them the next day and if queenless, a queen will be put into a colony; 5) Many robbing bees attacked FT-3; 6) In order to prevent robbing, dilute sugar syrup was sprayed every colony attacked by robbing bees after sunset.
2014/10/28	1) Each queen was colored as follows on October 26th: White for CR-1, 2, 3; red for DF-1, 2, 3; blue for CR-1, 2, 3; green for FT-1 ,2 , 3; 2) Chalk brood were found in CR-2; 3) Each queen of DF-2 and FT-3 was in a cage. A queen of DF-2 was released from the cage into the beehive after taking photos. A queen of FT-3 will be released into the beehive soon; 4) We could not find a queen in FT-1. We intended to put a queen soon.
2014/10/30	1) Chalk brood was found in CR-2; 2) The queen cells in FT-1 and FT-3 were removed; 3) We could not find a queen in FT-1 and found queen cells. So, we intended to put a new queen in a cage into FT-1 the next day (October 31st); 4) A queen in a cage was released into each beehive of FT-3 (November 2nd) and FT-1 (November 3rd)
2014/11/20	1) Chalk brood was found in CD-3 and FT-3; 2) Bees in CR-1 are a little more aggressive than the bees in CR-2 and CR-3; 3) As sugar syrup in the containers of DF-3, CN-3, FT-1 and FT-3 became almost empty and could not be fed to the colony, sugar syrup (50/50 by weight for sugar/water) was refilled at a given pesticide concentration in each container after removing the remainder of sugar syrup from the almost empty container on November 25th. Each remainder of sugar syrup (weight of non-consumed sugar syrup) was as follows: 301 g for DF-3; 227 g for CN-3; 215 g for FT-1; 287 g for FT-3.
2014/12/10	As the feeder tube of sugar syrup from the CN-1 container was clogged, it was fixed.
2014/12/29	1) There had been heavy wind for the last 4 days. There was no damage to the research colonies; 2) Control colonies (CR-1, 2, 3) were becoming more active; 3) We could not find the queen in CR-3 but found it in the next observation.
2015/1/17	1) We could not find the queen in CN-3 but could found eggs; 2) Macadamia tree was starting to flower.
2015/1/30	1) We could not find the queen and the eggs in CN-3 and could found a queen cell which seemed to have been capped at least five days before; 2) As sugar syrup in the containers of CN-1, CN-2 and FT-2 became almost empty and could not be fed to the colony, sugar syrup (50/50 by weight for sugar/water) was refilled at a given pesticide concentration in each container after removing the remainder of sugar syrup from the almost empty container on February 6th. Each remainder of sugar syrup (weight of non-consumed sugar syrup) was as follows: 247 g for CN-1; 199 g for CN-2; 120 g for FT-2.
2015/2/18	1) Control colonies (CR-1, 2, 3) 1) were suspicious of chalk brood; 2) DF-1 became extinct. The remainder of sugar syrup in the container of the extinct colony (DF-1) was measured; 3) DF-2 has no queen, no eggs and no queen cell and has many small hive beetles.

Table 5: Conts. Remarks in experimental observation.

2015/3/11	1) We discontinued conducting the experiment because of the rain after taking photos of CR-1, 2, 3 and DF-1,2 3; 2) Spotty brood were found on the first and second frames of CR-1; 3) Chalk brood were found on the front of the first frame (1F) of CR-2; 4) Drone cells were found on the front of the second frame (2F) of CR-3; 5) A queen cell was found in DF-2, whose size was small but more than 5 days old; 6) Perforated comb was found in the second frame; 7) Unwanted combs were found on the third frame; 8) Brood was still spotty. The suspicion is chalk brood due to rainy weather.
2015/3/14	1) We conducted the experiment which had been left due to the aborted experiment on March 11th and took photos of CN-1, 2, 3 and FT-1, 2, 3; 2) Spotty brood were found on the first and second frames of CN-1; 3) Spotty brood were found in CN-2; 4) Spotty brood were found in FT-1; 5) Hive beetle's eggs were found in FT-1; 6) Spotty brood were found in FT-2; 7) Unwanted combs were found on the third frame of FT-2; 8) Brood was still spotty. The suspicion is chalk brood due to rainy weather; 9) CN-3 became almost extinct; 10) The entrance of a beehive where the colony had become extinct (CN-3) was shut because of preventing other colonies from robbing the honey in the extinct colony. Hereafter we will shut the entrance of the beehive of the extinct colony.
2015/3/24	1) Spotty brood were found on the first, second and third frames of CR-1; on the first and second frames of CR-3; on the first, second and third frames of CN-1; on the second and third frames of CN-2; on the first, second and third frames of FT-1; on the second frame of FT-2; on the first, second and third frames of FT-3; 2) Bees of CR-3 were jumpy; 3) DF-2 became almost extinct; 4) Perforated brood cells were found on the first and second frames in DF-3; 5) Bees built own comb next to the fourth frame in FT-3; 6) As sugar syrup in the containers of CR-1, CR-2, CR-3, DF-3, CN-1, CN-2, FT-1, FT-2 and FT-3 became almost empty and could not be fed to the colony, sugar syrup (50/50 by weight for sugar/water) was refilled at a given pesticide concentration in each container after removing the remainder of sugar syrup from the almost empty container. Each remainder of sugar syrup (weight of non-consumed sugar syrup) was as follows: 430 g for CR-1; 205 g for CR-2; 0 g for CR-3; 1920 g for DF-3; 3420 g for CN-1; 8225 g for CN-2; 2535 g for FT-1; 1595 g for FT-2; 545 g for FT-3;
2015/4/24	1) FT-1 and DF-2 became extinct and DF-2 wax moths and wax-moth larvae were found in FT-1; 2) After taking photos, a pesticide was discontinued to be administered into every colony (a sugar syrup container with or without the pesticide was removed from every colony); 3) Chalk brood in CR-1 appears to be gone; 4) Spotty brood were still found, in CR-1 and DF-3, but looking better; 5) Spotty brood were found in CR-3, CN-1, FT-3; 6) Bees built own combs in top box of FT-3 (A few frames should be added); 7) We did not find the queen of DF-3 and CR-2 but found lots of eggs in DF-3; 8) We decided to discontinue feeding sugar syrup with a given pesticide concentration after considering that the amount of a pesticide consumed by each colony was enough to judge its effect on a honeybee colony. The remainder of sugar syrup in each container was measured after we remove the container from each beehive.
2015/5/11	1) CR-2 became extinct and wax moths & wax-moth larvae were found in CR-2. 2) Brood in CR-1 return to normal and chalk brood appears to be gone; 3) Honey was storing in CR-3; 4) New combs were built in FT-3 and brood are nice in FT-3; 5) The queen of CN-1 is slight.
2015/6/2	1) CR-2, DF-1, DF-2, CN-2, CN-3 and FT-1 has already become extinct; 2) Bees in DF-3 are jumpy and seem unorganized; 3) The queen of DF-3 is alive, but she seems to be failing; 4) Brood in FT-2 are spotty (unorganized behavior); 5) New combs were built in FT-3 and honey and nectar are stored. FT-3 is vigorous.
2015/7/20 finish of experiment only visual observation	1) Experiment was finished and we did not take photos but jotted down the points of observation; 2) DF-3 and CN-1has already become extinct; 3) So far all of colonies where neonicotinoids were administered (DF-1,2,3 and CN-1,2,3), one of colonies where organophosphates were administered (FT-1) and one of control colonies have become extinct.
2015/10/15 only visual observation	1) We did not take photos but jotted down the points of observations in our research note; 2) CR-3 became extinct. After swarming, the rest of the CR-3 colony became weak and finally extinct.

Table 5: Conts. Remarks in experimental observation.

2015/11/5 visual observation	1) We did not take photos but jotted down the points of observations in our research; 2) The queen in FT-2 was lost, adult bees were 200 to 300 only (robbing bees?), capped brood were 5 to 10 only, no eggs, no larvae , no dead bees no small hive beetles (the effect of fenitrothion?), no honey (no nectar), no pollen were, and combs were clean but spotty (Such tendency began to be shown from the middle of October). That is, FT-2 became almost extinct; 3) FT-3 which was composed of 7 frames was very active.

Table 6: Number of dead bees.

Date	Elapsed [day]	CR-1	CR-2	CR-3	DF-1	DF-2	DF-3	CN-1	CN-2	CN-3	FT-1	FT-2	FT-3
22-Oct-14	0	0	0	0	0	0	0	0	0	0	0	0	0
23-Oct-14	1	0	0	0	0	0	0	0	0	0	0	0	0
24-Oct-14	2	4	3	5	69[1)]	4	7	15	14	5	9	4	33
25-Oct-14	3	2	0	2	16[2)]	6	7	0	1	24	2	2	5
28-Oct-14	6	0	10	0	10	0	0	3	0	11	2	0	0
30-Oct-14	8	1	16	2	9	0	2	1	1	4	2	1	4
20-Nov-14	29	1	7	3	40	0	2	0	1	0	3	0	1
10-Dec-14	49	4	12	7	120	1	4	3	9	4	4	7	7
29-Dec-14	68	6	3	2	25	3	7	0	5	1	0	1	13
17-Jan-15	87	17	13	18	12	2	1	3	2	3	6	2	0
30-Jan-15	100	3	0	2	8	6	3	3	2	2	1	1	2
18-Feb-15	119	27	3	8	0	6	22	2	7	28	4	3	6
11-Mar-15	140	61	19	47		5	73						
14-Mar-15	143							12	14	38	8	2	10
24-Mar-15	153	14	23	6		7	2	6	2		6	9	2
4-Apr-15	164	8	7	30		10	1	0	0		63	0	4
11-May-15	201	2	125	72			0	0	2			0	0
2-Jun-15	223	1		5			0	2				0	0

1) DF-1 colony has been attacked by many hive-beetles. The comb badly damaged will be replaced; 2) The comb badly damaged has been replaced with a new comb with similar number of brood produced by a sister queen to the previous one.

suddenly become extinct in mid-May and CR-3 continues to slightly increase in number. Examining the dinotefuran colony group (DF-1, DF-2 and DF-3) where dinotefuran was administered, we can find that DF-1 and DF-2 have become extinct before April without passing through a process of an increase in the number of adult bees and with those remaining at a low level. On the other hand, DF-3 existed till June but thereafter was found to have already become extinct in late July after passing through a process of the general change in the number of adult bees while keeping the number at higher levels from the start of experiment. Examining the clothianidin colony group (CN-1, CN-2 and CN-3) where clothianidin was administered, we can find that CN-1 existed till June but thereafter was found to have already become extinct in late July after passing through a similar process to the general change in the number of adult bees under a lower level of an increase in number from March than the general increase such as CR-1, DF-3 and FT-3. Thereafter, CN-2 and CN-3 have become extinct in the beginning of June and in mid-March and without a rapid increase in the number of adult bees, respectively. Examining the fenitrothion colony group (FT-1, FT-2 and FT-3) where clothianidin was administered, we can find that FT-2 and FT-3 survived as well as, the control colonies, CR-1 and CR-3, while showing the general change in the number of adult bees estimated by Apao (2014). Especially, FT-3 was most active of all colonies and FT-1 became extinct at the beginning of April after a slight increase in the number.

Number of capped brood

Table 8 shows the number of capped brood in each colony, while Figure 6 illustrated it. Figure 6 demonstrates that

Table 7: Number of adult bees.

Date	Elapsed day	Number of adult bees											
		CR-1	**CR-2**	**CR-3**	**DF-1**	**DF-2**	**DF-3**	**CN-1**	**CN-2**	**CN-3**	**FT-1**	**FT-2**	**FT-3**
22-Oct-14	0	3287	5031	6109	1903	1580	2199	2890	2094	3658	3317	2261	7277
23-Oct-14	1	4584	5507	6230	2147	1517	2949	2885	2195	4096	3680	2730	7716
24-Oct-14	2	5341	5327	8131	1889	1405	4130	2809	2212	4385	4407	2737	8517
25-Oct-14	3	4778	4964	6904	1547	1391	4469	2798	2157	4204	4454	2752	7666
28-Oct-14	6	5017	4665	8244	1814	2116	5788	3553	3272	4949	4669	3870	8431
30-Oct-14	8	5311	5153	7778	1908	2348	5515	3497	3050	4880	4732	3748	8142
20-Nov-14	29	5845	3663	6200	1576	2272	4923	3189	2960	4259	4259	3476	4319
10-Dec-14	49	4619	2501	4094	1074	1979	4145	2852	1999	3685	3908	2903	3466
29-Dec-14	68	3569	1619	2978	1202	1810	3818	2935	2080	3349	3427	2233	2837
17-Jan-15	87	2922	1348	2186	711	1620	3823	2855	1806	3243	3389	2098	2628
30-Jan-15	100	3275	1336	2221	495	1580	3735	2614	1917	2871	3009	2369	2641
18-Feb-15	119	4261	1683	2550	0	1714	4361	4074	2361	1388	3032	3087	4024
11-Mar-15	140	6989	2341	4003		933	7454	5911	3367	201	5290	5037	7507
14-Mar-15	143	7534	2498	4178		793	7991	6173	3511	31	5612	5315	8005
17-Mar-15	146	8079	2654	4352		653	8529	6139	3570	0	5543	5566	9231
24-Mar-15	153	9350	3024	4760		326	9783	6060	3709		5381	6150	12091
4-Apr-15	164	10857	2916	6476		0	11600	5350	3334		0	5512	12820
11-May-15	201	13928	516	5033			12398	8573	5179			10660	19609
2-Jun-15	223						9433	6523	0				
20-Jul-15	271						0	0					

Note: Experiment started on October 22nd 2014 and a pesticide was administered from October 23rd 2014 to April 4th 2014.

capped brood shows a roughly similar change in number to the number of adult bees though it varies in number more widely than adult bees as follows: capped brood increase rapidly from the latter half of February, 2015 after they decrease slightly from the start of experiment to the latter half of January. Examining the control colony group (CR-1, CR-2 and CR-3) in few details, CR-1 and CR-3 shows a similar change to the general change in the number of the adult bees in Maui shown in Figure 5, though CR-3 increased in the number of caped brood more slowly than CR-1. CR-2 became extinct in mid-May without an increase in the number. Examining the dinotefuran colony group (DF-1, DF-2 and DF-3), we observed that DF-1 and DF-2 became extinct in late February and March almost without an increase in the number of capped brood as in the case of the number of adult bees, respectively. On the other hand, DF-3 became extinct in late July after capped brood in DF-3 changed with time in a complicated manner where the number in DF-3 repeats a small increase and decrease from the start of experiment to late January and then began to increase sharply from late February which later decreased at the beginning of April and thereafter began to increase sharply again but later decreased; finally, DF-3 became extinct in late July.

Examining the clothianidin colony group (CN-1, CN-2 and CN-3), we observed that CN-1 became extinct on late July after a somewhat delayed sharp increase where the number in CN-1 repeats a slight increase and decrease from the start of experiment to the beginning of April, thereafter, it sharply increased to the mid- May and then decreased and finally CN-1 became extinct. CN-2 became extinct at the beginning of June after giving an indication of a small increase in the number of capped brood. CN-3 became extinct in mid-March after the sharp increasing period of the number of capped brood. Such tendency is very similar to the number of adult bees.

Examining the fenitrothion colony group (FT-1, FT-2 and FT-3), we observed that FT-2 and FT-3 survived to the end of the experiment as well as, the control colonies, CR-1 and CR-3. FT-3 is most active of all colonies including the control colonies for an experimental period. FT-1 began to increase in the number of capped brood in the latter half of February but thereafter decreased in the number of capped brood and thereafter, FT-1 became extinct at the beginning of April.

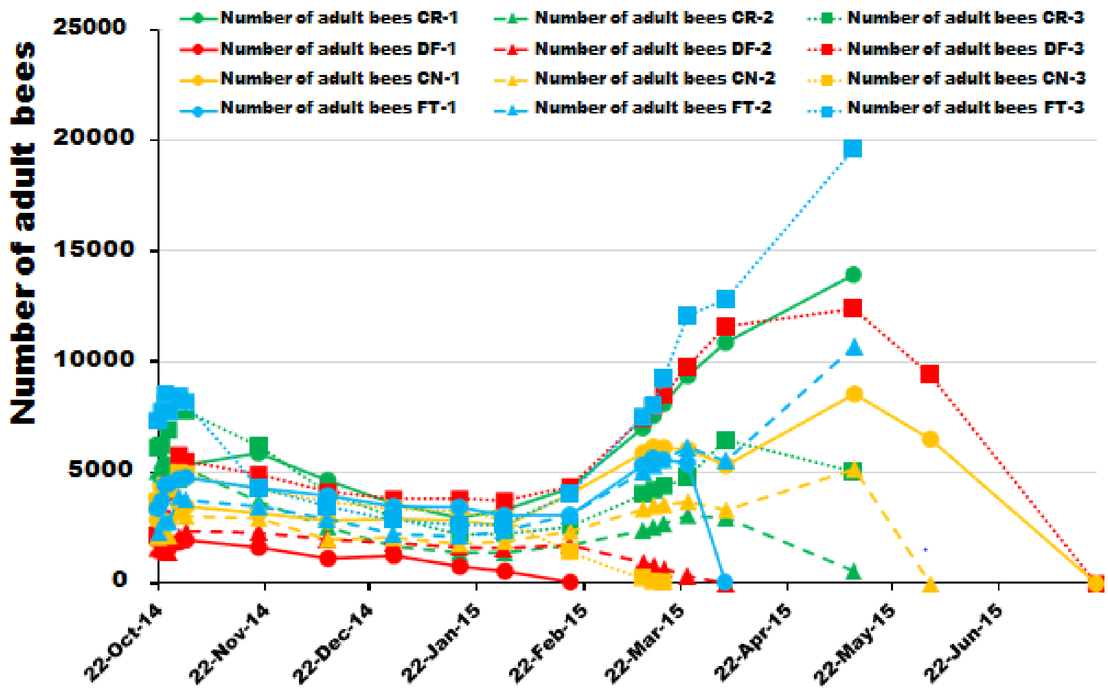

Figure 4: Change in the number of adult bees in each colony.

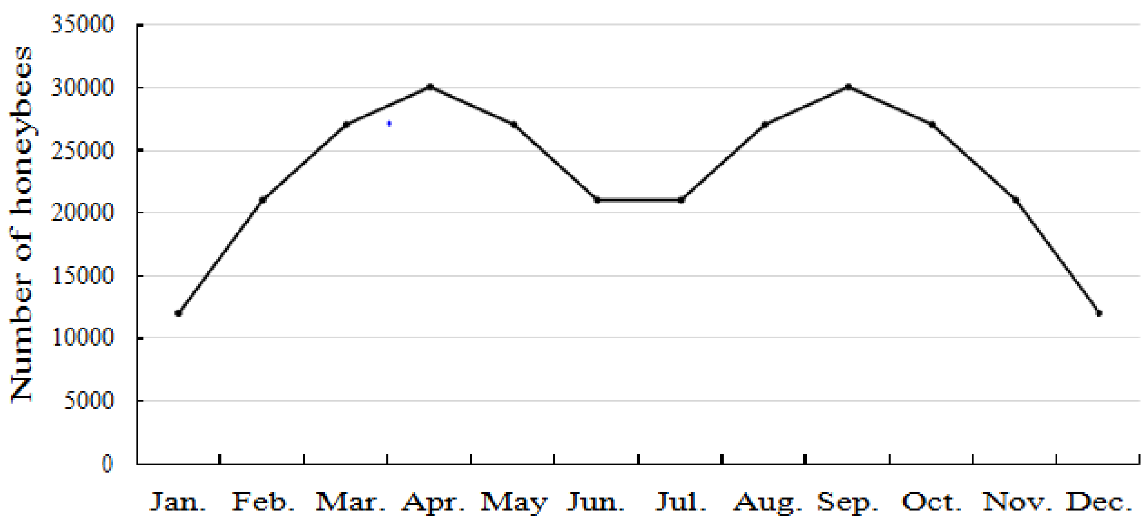

Figure 5: Seasonal change in number of honeybees in an average colony in Maui given by Paul (2014).

It was confirmed that the number of capped brood changes with time very similar to that of adult bees. We estimated that a flowering season and/or the weather seem to be deeply concerned in the changes in the numbers of adult bees and capped brood.

Intake of pesticide

We examined the amount of a pesticide which is consumed by honeybees from two viewpoints of the intake of pesticide ingested by a colony and that by a honeybee because an impact of a pesticide on a large colony seems to be lower than that of a small colony when the same amount of a pesticide is consumed by a colony.

Total intake of pesticide consumed by a colony

The total intake of pesticide consumed by a colony

Table 8: Number of capped brood.

Date	Elapsed day	Number of capped brood											
		CR-1	**CR-2**	**CR-3**	**DF-1**	**DF-2**	**DF-3**	**CN-1**	**CN-2**	**CN-3**	**FT-1**	**FT-2**	**FT-3**
22-Oct-14	0	1144	1648	2595	2267	1832	3775	1474	2203	2386	4103	2632	1284
23-Oct-14	1	1186	1442	2620	2203	1856	3240	1371	2068	2370	3728	2445	1050
24-Oct-14	2	1204	1585	2649	1880	1880	3929	1388	1890	2269	2988	2191	806
25-Oct-14	3	1274	1634	2635	1746	1639	3191	1403	1605	2083	2222	1954	600
28-Oct-14	6	1691	1765	2157	1395	1195	1386	1225	533	1606	918	799	114
30-Oct-14	8	2029	1638	2605	1253	1065	956	1000	354	1559	827	513	89
20-Nov-14	29	1154	1111	2016	792	1520	2457	1454	1403	2138	1501	1854	1676
10-Dec-14	49	1290	462	849	345	782	944	1373	827	1194	1058	751	1213
29-Dec-14	68	1409	371	828	366	659	1946	918	712	1342	1000	693	1066
17-Jan-15	87	1043	398	1019	200	575	1557	529	1024	1550	108	644	1005
30-Jan-15	100	2215	528	1299	209	781	1621	1984	1240	763	587	1913	1926
18-Feb-15	119	2876	908	1566	17	1153	2518	2644	1636	26	2190	2564	3042
11-Mar-15	140	4974	954	2894		8	5412	1727	2231	3	1653	3079	6458
14-Mar-15	143	5062	949	3074		6	5311	1596	2316	0	1576	3153	6946
17-Mar-15	146	5150	852	3253		4	5211	1333	1975	0	1501	2533	6970
24-Mar-15	153	5355	734	3672		0	4976	719	1178		1326	1086	7027
4-Apr-15	164	5897	1722	3866		0	3514	2572	2532		107	4043	7023
11-May-15	201	6831	3	3671			7715	7001	2688			8555	9540
2-Jun-15	223						5870	5327	0				
20-Jul-15	271						0	0					

Note: Experiment started on October 22nd 2014 and a pesticide was administered from October 23rd 2014 to April 4th 2014.

(hereafter, the total intake of pesticide per colony) is one of the indicators to assess the magnitude of the pesticide impact on a honeybee colony, which can be easily obtained from a difference between the administered amount of a pesticide into a colony and the remaining amount of a pesticide. Table 9 shows the total intake of each pesticide per colony during the pesticide administration period from the start of administration of a pesticide (October 23rd 2014) to the stop of administration April 4th 2015 or to the point in time when the colony became extinct before April 4th 2015 and the total amount of sugar syrup consumed by a colony during the pesticide administration period (hereafter, the total consumption of sugar syrup per colony). Figure 7 shows the total intake of each pesticide per colony during the pesticide administration period. Where a pesticide was administered into a colony from October 23rd 2014 to April 4th 2015 or to the point in time when the colony became extinct before April 4th 2015. It was observed from Table 9 and Figure 7 that there is a considerable difference in the pesticide intake per colony among same pesticide group and a great difference in the pesticide intake per colony among different groups. The difference in the pesticide intake per colony among the same pesticide group may possibly come from the differences in colony size and survival period and that among different groups seem to come from the result that a concentration of each pesticide was prepared so as to have the same insecticidal activity to exterminate stinkbugs among pesticides.

Examining the difference in the pesticide intake per colony among the same pesticide group in a little more detail, there are great differences in the pesticide intake more than ten times among the dinotefuran colonies (DF-1, DF-2 and DF-3) all of which became extinct; there are a few differences

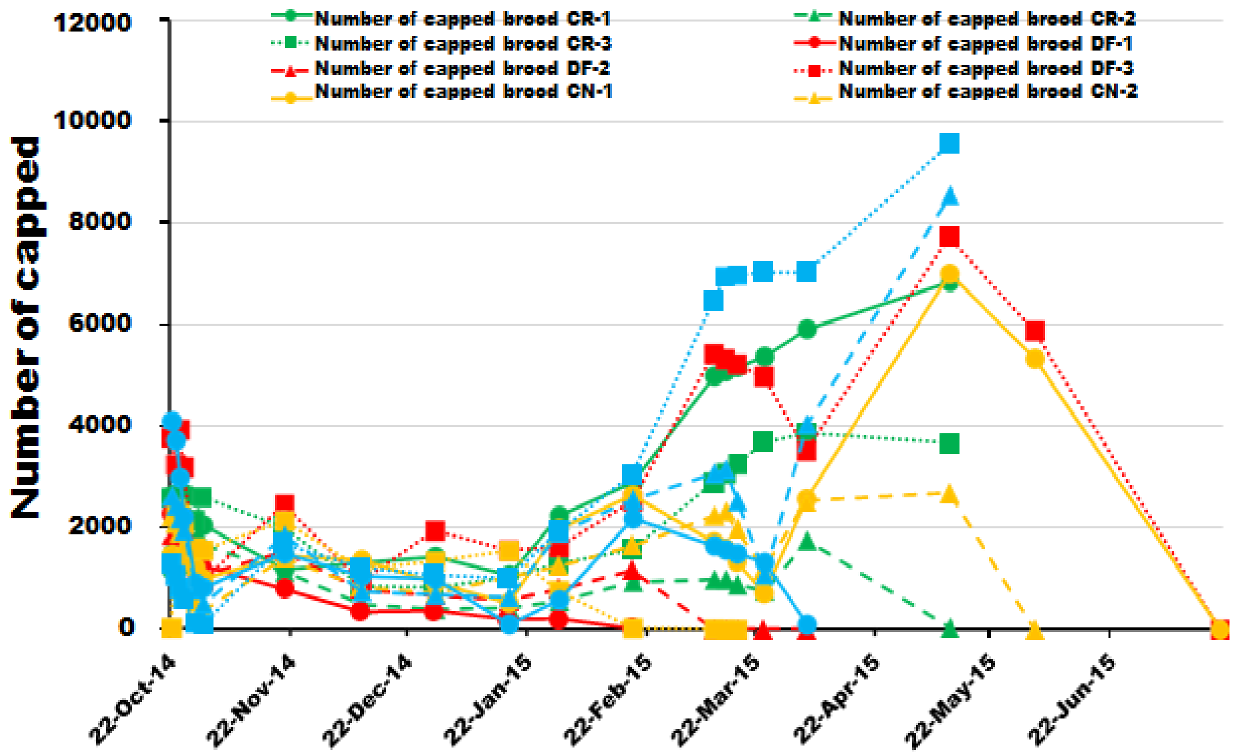

Figure 6: Change in the number of brood in each colony.

Table 9: Total intake of pesticide per colony.

Name of colony	Kind of pesticide	Concentration of pesticide [ppm]	Total consumption of sugar syrup [kg]	Total intake of pesticide [mg]	State of colony at the end of experiment
CR-1			11.57	0	Active
CR-2	Pesticide free	0	11.795	0	Extinct
CR-3			12	0	Active
DF-1			1.377	0.2754	
DF-2	Dinotefuran	0.2	11.905	2.381	Extinct
DF-3			44.924	8.9848	
CN-1			26.213	2.097	
CN-2	Clothianidin	0.08	18.531	1.4825	Extinct
CN-3			17.573	1.4058	
FT-1			27.18	27.18	Extinct
FT-2	Fenitrothion	1	28.08	28.08	Active
FT-3			46.338	46.338	

among the clothianidin colonies (CN-1, CN-2 and CN-3) all of which became extinct and there are some differences among the fenitrothion colonies (FT-1, FT-2 and FT-3) where only FT-1 became extinct and ingest the least fenitrothion of all the fenitrothion colonies. Only CR-2 became extinct even among the control colony group where no pesticide was administered.

The intake of pesticide per colony is converted into the amount of clothianidin equivalent to the toxicity of each pesticide in order to examine the difference in the pesticide intake per colony among the different groups as shown in Figure 8. It can be seen from Figure 8 that there is a

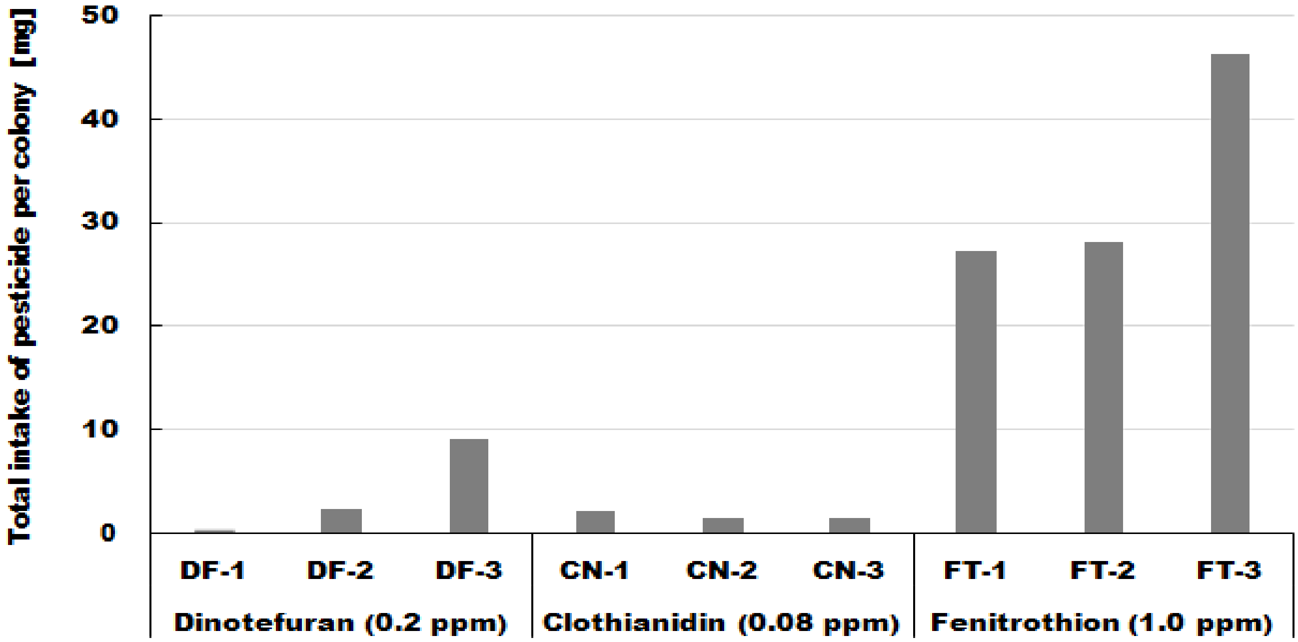

Figure 7: Total intake of pesticide per colony to the colony extinction or to the finish of experiment.

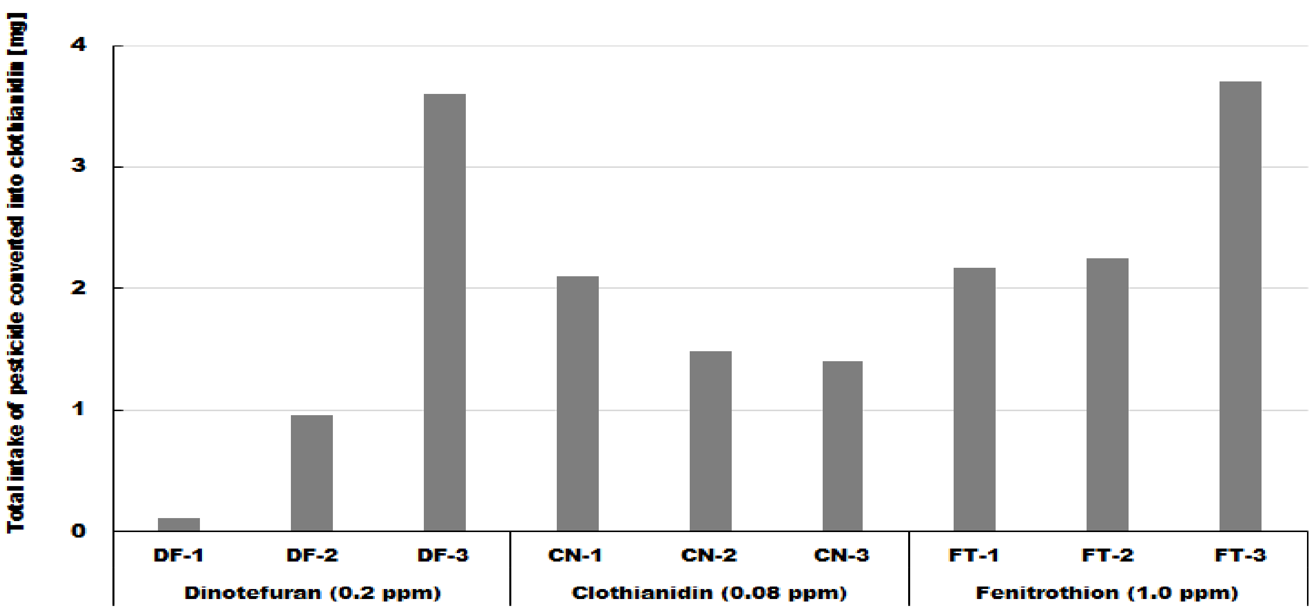

Figure 8: Total intake of pesticide converted into clothianidin per colony. On the assumption that each pesticide has the same insecticidal activity to exterminate stinkbugs when each pesticide concentration satisfies the following relationships, that is, 0.08 ppm of clothianidin, = 0.2 ppm of dinotefuran = 1 ppm of fenitrothion, each pesticide intake is converted into clothianidin intake.

relatively small difference in the converted intake into clothianidin among the averages in each colony group though there is a great difference in the converted intake among the dinotefuran colonies. We further discussed the causes of the great difference among the same pesticide group and the reasons why pesticide-free CR-2 and FT-1 with the least fenitrothion intake have become extinct.

Intake of pesticide ingested by a honeybee

An impact of a pesticide on a honeybee colony seems not always to be able to be correctly assessed with the total intake of pesticide per colony dependent on the colony size and the survival period of colony. Therefore, we tried to obtain the intake of pesticide ingested by a honeybee

Table 10: Intake of pesticide per bee till colony extinction or end of experiment.

Kind of pesticide	Name of colony	Total intake of pesticide1) [mg]	Total number of adult bees2)	Intake of pesticide per bee [ng/bee]	State of colony at the end of experiment
Dinotefuran (0.2 ppm)	DF-1	0.2754	8359.3	32.95	Extinct
	DF-2	2.381	13001.2	183.14	
	DF-3	8.9848	79370.7	113.2	
Clothianidin (0.08 ppm)	CN-1	2.097	61267.9	34.23	Extinct
	CN-2	1.4825	28253.8	52.47	
	CN-3	1.4058	18491.2	76.03	
\ Fenitrothion (1.0 ppm)	FT-1	27.18	20039.8	1356.3	Extinct
	FT-2	28.08	82594.1	339.98	Active
	FT-3	46.338	108276.5	427.96	

1) Total intake of pesticide means the total intake of pesticide from the start of pesticide administration (October 23rd 2014) to the colony extinction or to the discontinuation of administration (April 4th 2015); 2) Total number of adult bees means the total number of adult bees from the start of pesticide administration (October 23rd 2014) to the colony extinction or to the end of experiment (July 20th 2015).

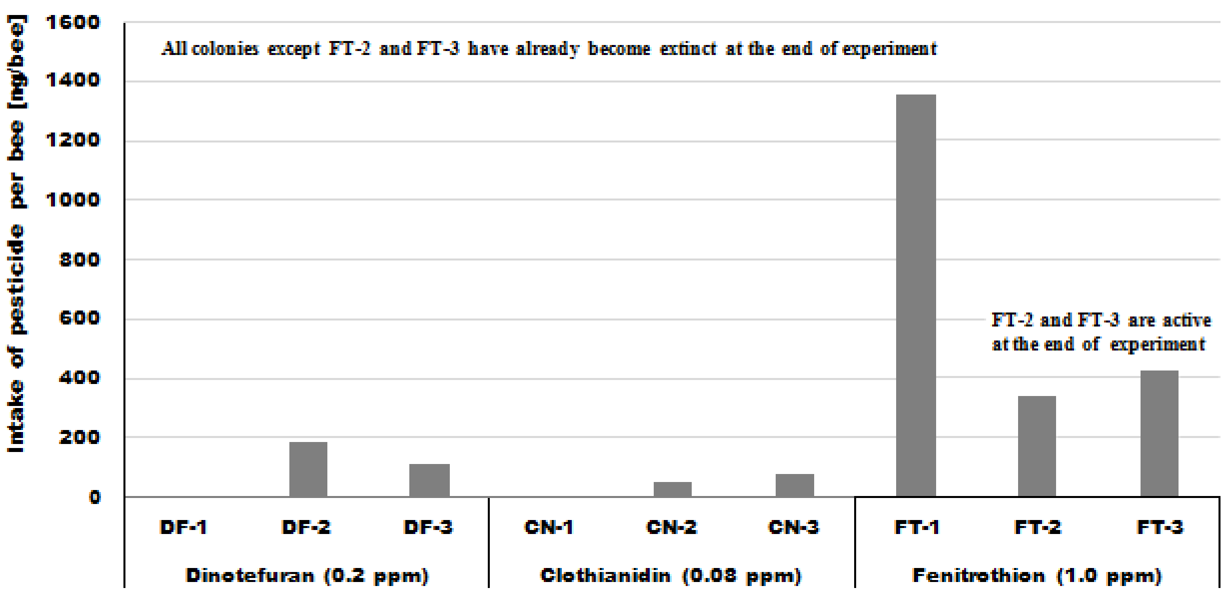

Figure 9: Intake of pesticide per bee to the colony extinction or to the end of experiment. The intake of pesticide per bee can be obtained by dividing the total intake of pesticide per colony by the total number of honeybees according to the procedures reported in our previous works (Yamada et al., 2018a, b, c).

(hereafter, the intake of pesticide per bee). The intake of pesticide per bee is one of indicators to assess the magnitude of the pesticide impact on a honeybee, which can be obtained from the total intake of pesticide per colony and the total number of adult bees during pesticide administration. In this work, a pesticide was administered into a colony from October 23rd 2014 to the extinction of colony or to April 4th 2015 when the colony survived. Table 10 shows the total number of adult bees during the administration of a pesticide and the intake of pesticide per bee, which are obtained from the procedures shown in Yamada et al. (2018a, b, c). Figure 9 shows the intake of pesticide per bee during the administration of a pesticide. It can be seen from Figure 9 that the fenitrothion colony group, the dinotefuran colony group and the clothianidin colony group are roughly ranked in order of the intake of pesticide per bee.

Examining the intake of fenitrothion per bee in the fenitrothion colonies in a few details, the intake of fenitrothion per colony in FT-3, which is shown in Table 9 and Figure 7 is the most of all the fenitrothion colonies but the intake per bee in FT-3 becomes much lower than that in FT-1 as shown in Figure 9. One of the reasons why there is such a big difference in the intake between per colony and

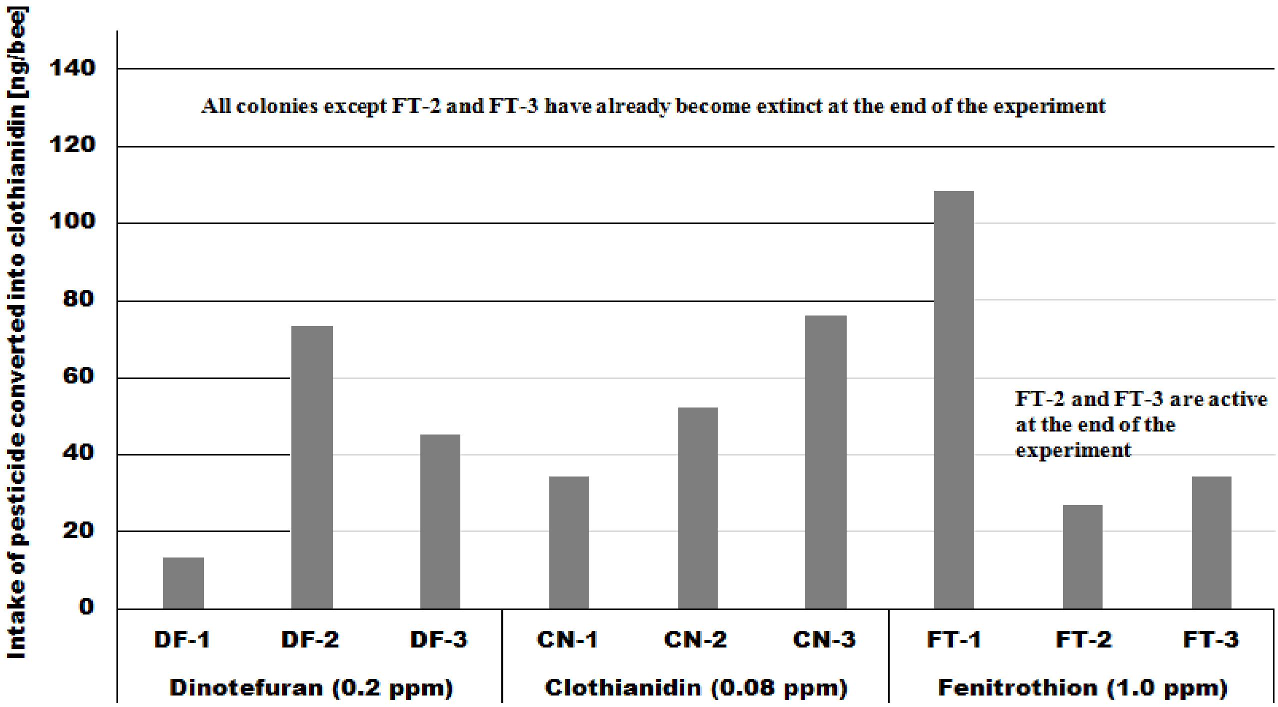

Figure 10: Intake of pesticide converted into clothianidin per bee to the colony extinction or to the end of the experiment.

per bee seems to be because FT-3 which is very active has much more bees than the other fenitrothion colonies and therefore a ratio of house bees which ingest sugar syrup in a hive to foraging bees which go out foraging become much lower than the others though the total number of adult bees is much higher than the others. We can apply a similar reasoning earlier mentioned in the case of DF-3 because the intake of dinotefuran per bee in DF-3 whose intake of dinotefuran per colony is the most of the entire dinotefuran colony became fewer than that in DF-2.

In order to compare the difference in the intake of pesticide among the different pesticide groups, the intake of each pesticide per bee during the pesticide administration is expressed after being converted into clothianidin as shown in Figure 10. We observed from Figure 10 that the converted intake of pesticide per bee into clothianidin varies widely among the same pesticide group; that is, a maximum converted intake of dinotefuran per bee into clothianidin is about 5.6 times as much as, a minimum converted intake among the dinotefuran colonies; a maximum intake of clothianidin per bee is about 2.2 times as much as, a minimum intake among the clothianidin colonies, and a maximum converted intake of fenitrothion per bee into clothianidin is about 4 times as much as a minimum intake among the fenitrothion colonies. On the other hand, only a slight difference in the average pesticide intake per bee among the same pesticide colony group is recognized among the different pesticide colony groups; an average intake of 44 ng/bee among three dinotefuran colonies; an average intake of 54 ng/bee among three clothianidin colonies and an average intake of 57 ng/bee among three fenitrothion colonies.

Measurement of temperature and humidity around and in a hive

Figure 11 shows the changes in temperature and humidity on the outside of the hive of CR-1 (at the corner of the southwest in the experimental site) and on the insides of the hives of CR-2, DF-2, CN-2 and FT-2 from October 15th to November 15th or to the point in time when the colony became extinct, which were continuously recorded on a data logger. We can understand from Figure 11 that the daily inside temperature variation of a hive is smaller than the outside temperature variation but the mean daily inside temperature of a hive is higher than that on the outside all year round; the daily inside humidity variation of a hive is smaller than the outside humidity variation all year round. From the results obtained it is concluded that the inside variations in temperature and humidity of a hive seem to be smaller than the outside variations in temperature and humidity.

DISCUSSION

Extinction rate of colony

The extinction rates of colony on July 20th 2015 are about 33% in the control colony group (CR-1, CR-2 and CR-3),

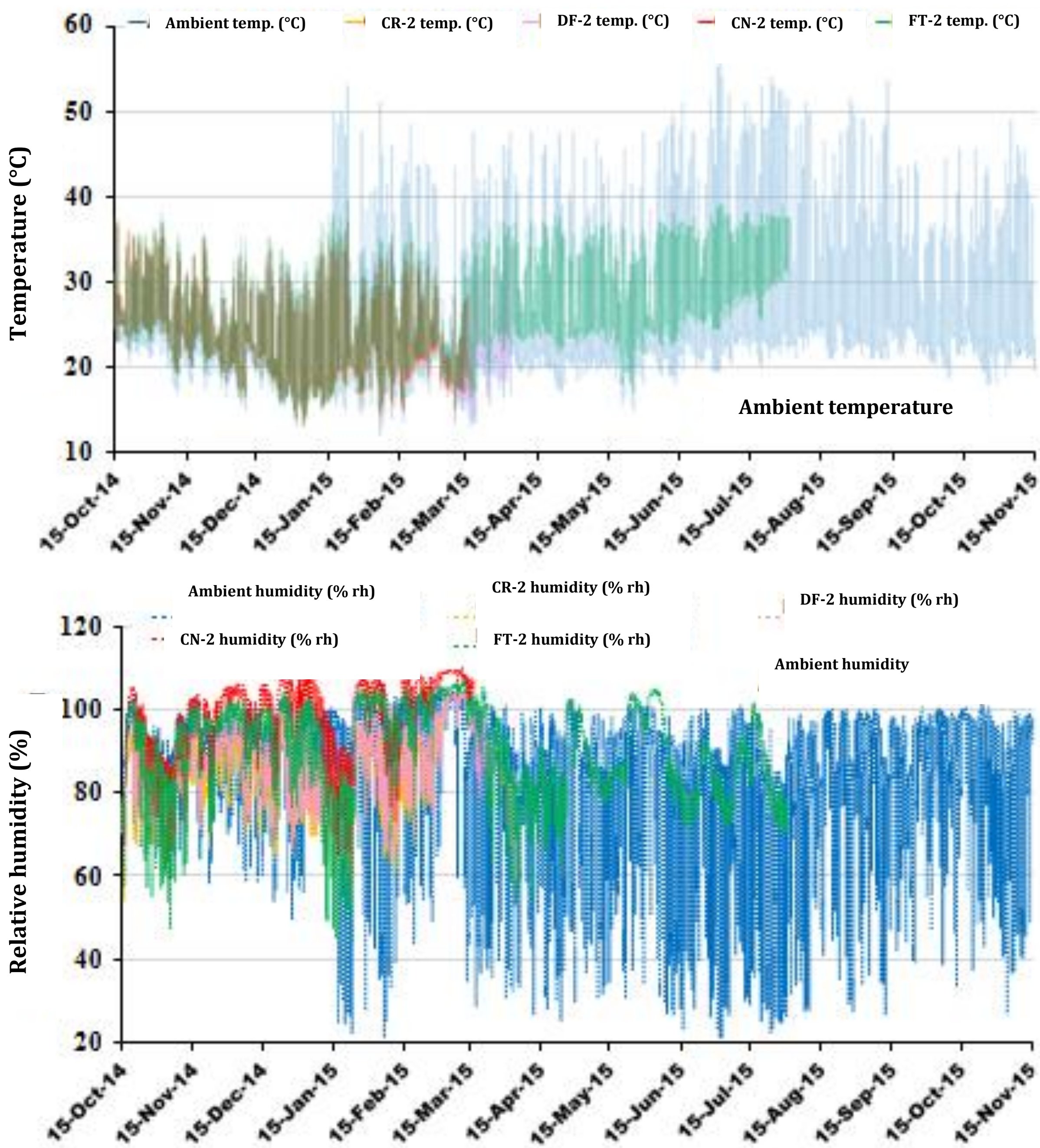

Figure 11: Seasonal change in temperature and humidity in each hive and the ambience in the experimental site in Maui (Paul, 2014).

100% in the neonicotinoid (dinotefuran and clothianidin) colony group (DF-1, DF-2, DF-3, CN-1, CN-2 and CN-3) and 33% in the organophosphate (fenitrothion) group (FT-1, FT-2 and FT-3) as well as, the control colony group (Table 11). It is clear that the extinction rate of the neonicotinoid colony group is much higher than those of the control and organophosphate colony groups. This result supports our previous works (Yamada et al., 2018b, c).

Currently, we discussed the reasons for the notable difference in extinction rate between the neonicotinoid colony group and others. One possible reason for this may be that a neonicotinoid pesticide has a few distinguished characteristics, such as a long-term persistency that it is very stable and its toxicity is often kept even after the pesticide has decomposed and a strong toxicity which is about five times as strong as an organophosphate pesticide, etc. The following scenario is considered from the characteristics of a neonicotinoid earlier mentioned. In this experimental site surrounded by vast pesticide-free areas, honeybees ingest a pesticide directly through toxic sugar

Table 11: Extinction rate and survival rate of honeybee colony at the finish of field experiment on July 20th 2015.

Pesticide		Extinction rate (%)	Survival rate (%)
Control	Pesticide-free	33	67
Neonicotinoid	Dinotefuran and Clothianidin	100	0
Organophosphate	Fenitrothion	33	67

syrup with a pesticide and/or indirectly through toxic honey and toxic bee bread stored in cells on combs. Though only a small difference in the intake per bee seems to be made between a neonicotinoid pesticide and an organophosphate pesticide, a notable difference in extinction seems to be caused by the difference in persistency between both. Honeybees convert nectar and sugar syrup into honey and then store a part of honey in cells of a comb. The amount of honey stored depends on the conditions in blooming. In the blooming (nectar flow) season honeybees directly ingest nectar in preference to sugar syrup and store sugar syrup in cells on a comb mainly as honey or partly as bee bread. Honeybees, therefore, will ingest sugar syrup converted into stored honey when there are few flowers in bloom. As a neonicotinoid pesticide, such as dinotefuran and clothianidin, which stays effective for a long period of time after being sprayed continues to affect adversely a honeybee colony through toxic honey and toxic bee bread which are stored in a hive for a substantial period of time not only when being sprayed but also even after being sprayed, it will be more prone to collapse a honeybee colony than the other pesticides with short-term persistency.

On the other hand, as an organophosphate pesticide which is much more unstable and much weaker in toxicity than a neonicotinoid pesticide is effective only for a very short period of time; its toxicity is rapidly weakened while sugar syrup containing an organophosphate pesticide is stored in a container and sugar syrup converted into honey is stored in a hive. An organophosphate pesticide such as fenitrothion will therefore have limited adverse effects on a honeybee colony except in cases where honeybees ingest plenty of the pesticide for a short period of time. Such a difference in pesticide persistence can result in the difference in extinction rate. If so, why did FT-1 where the organophosphate fenitrothion was administered become extinct on April 4th 2015? Here, we deduced the causes of the extinction of FT-1. A pesticide affects adversely not only adult bees but also brood and a queen and then causes an increase in dead bee, reduction in colony and oviposition, aberrant bees and eggs. Examining the number of capped brood in FT-1 shown in Table 8 and Figure 6 as evidence for the number of eggs (the ovipositional capacity), the numbers of capped brood in FT-1 (extinct) decreases from mid-February, although conversely those in FT-2 (surviving), and FT-3 (surviving) increase. Although FT-1 and FT-2 are about the same total intake of fenitrothion (ca. 27 mg in FT-1 and ca. 28 mg in FT-2), the total number of adult bees in FT-1 (ca. 20000) is only about one-fourth as that in FT-2 (ca. 83000) (Table 10). That is to say, FT-1 must have ingested a great deal of fenitrothion for a comparatively short period of time in comparison with FT-2. Ingestion of a great deal of fenitrothion weakens FT-1, reduces the colony size and the ovipositional capacity of a queen but it cannot cause collapse of FT-1 because the organophosphate fenitrothion weakens rapidly in toxicity depending on the time frame. Reductions in the queen's ovipositional capacity and the colony activity will permit insect pests such as wax-moth larvae to attack a colony (FT-1) when fenitrothion existing in a hive becomes ineffective. According to our experience, few wax-moth larvae can get into a hive where a pesticide is effective. When honeybees become uncomfortable in the hive after the hive teems with wax-moth larvae, honeybees and a queen will escape from the hive. This reason is based on the facts that there were several wax-moths and many wax-moth larvae in the hive of FT-1 when FT-1 became extinct as shown in Table 5 and FT-1 was relatively active just before the colony became extinct as shown in Table 7 and Figure 4.

At this juncture, we deduced the reasons why CR-2 where no pesticide was administered became extinct. Judging from the fact that the number of capped brood in CR-2 is kept fewer for a long period of time (Table 8 and Figure 6) and a few wax moth and some wax-moth larvae had been found in CR-2 before extinction, CR-2 seems to have escaped from the hive because of the attacks by pests such as wax-moth larvae.

As seen from the cases earlier mentioned, FT-1 and CR-2 seem to have escaped from the hive rather than become extinct because of uncomfortable conditions due to attacks by pests. It is concluded that the much higher extinction rate (100%) in the neonicotinoid colony group than those (33%) in the other groups seems to be due to the long-term persistency and high toxicity of inherent characteristics in a neonicotinoid pesticide, which continues to chronically affect adversely a honeybee colony for a long period of time even when its concentration is low and finally it may result in the extinction of a colony. On the other hand, a short-term persistence pesticide such as an organophosphate pesticide causes acutely massive death of honeybees just after the pesticide is administered (sprayed) but thereafter it will hardly affect adversely honeybees for a long period of time because it rapidly becomes ineffective and as a result cannot lead to colony extinction.

Intake of pesticide by honeybees

We encountered the following issues in order to obtain efficiently data with high accuracy in a minimum number of honeybee colonies when we assess and evaluate an impact of a pesticide on a honeybee colony in a long-term field experiment like: (1) Evaluation of the administered amount of a pesticide; which is better to evaluate and assess an impact on a honeybee colony, the total intake of pesticide consumed by a honeybee colony or the intake of pesticide ingested by a honeybee? (2) Evaluation of the difference in insecticidal activity among pesticides; how to make corrections and an evaluation for differences in insecticidal activity; (3) Evaluation of the intake of pesticide per bee in a surviving colony; how to evaluate and assess the intake of pesticide per bee and the impact on a surviving colony after discontinuation of pesticide administration into a honeybee colony.

Evaluation of the administered amount of a pesticide

In a long-term field experiment such as this experiment, the total intake of pesticide per colony can be directly obtained from the difference between the total amount of a pesticide which was administered during the experimental period and the remaining amount of a pesticide at the end of experiment. The total intake of pesticide per colony depends on the colony size (the numbers of adult bees, brood and eggs, the amounts of honey and bee bread stored in a hive), the colony activity (the numbers of house bees and foraging bees and the ovipositional capacity of a queen). It is very difficult to even off the colony size and activity among experimental colonies in a long-term field experiment. In order to offset the bad influence of the discrepancy in colony size and activity among experimental colonies on the intake of pesticide, it is necessary to statistically process the intakes of a pesticide obtained from an experiment with many colonies under the same condition. A great number of colonies demand an enormous cost and time and seems not to be a realistic solution in a long-term field experiment though they are effective for a laboratory experiment. If we can close the differences in colony size and activity among colonies, a small number of colonies can give effective results but to close the differences is very difficult and not feasible. It may be feasible to adjust the ratio of the maximum value to the minimum value for each number of adult bees and capped brood to be within a few and to select each queen having identical characteristic (the same origins). Then normalization of the number of adult bees in a certain colony to that in a control colony as $(n_{ij}/n_{i0})/(n_{cj}/n_{c0})$ as proposed by Yamada et al. (2012) seems to be effective for adjusting the difference among colonies when adult bees are more than several thousand because it makes it possible to compensate for both a difference in initial bee population among colonies and that in seasonal fluctuation of bee population, where n_{ij} is the number of adult bees in colony after the elapse of j days, n_{i0} is the initial number of adult bees in colony i at the start of experiment, n_{cj} is the number of adult bees in control colony after the elapse of j days, and n_{c0} is the initial number of adult bees in control colony at the start of experiment. One thing we should pay attention to is that this normalization method is based on a precondition that each member in polyethism is almost the same among colonies. In any case, the total intake of pesticide per colony will only give a comparison of the impact of a pesticide among colonies which are regarded as a super-organismic individual by neglecting the difference in colony size and activity among colonies.

On the other hand, the intake of pesticide per bee seems to be eliminated to some extent but not-perfectly. To obtain the intake of pesticide per bee, we need to measure the numbers of adult bees and capped brood with high accuracy and the total intake of pesticide per colony which can be accurately obtained from the experimental results. Yamada et al. (2018a, b, c) proposed one of the methods to obtain the intake of pesticide per bee using the numbers of initial adult bees and capped brood. In this work, we evaluated and assessed the impact of a pesticide on a bee colony by the intakes of a pesticide both per colony and per bee as shown in Figures 7 and 9, and Tables 9 and 10, pesticide was stopped to be administered into a colony when a colony became extinct, or on April 4th 2015 in the case that the colony survived, and thereafter pesticide-free sugar syrup was fed into a surviving colony till July 20th when the experiment was completed It was assumed that honeybees in a colony continued to ingest toxic sugar syrup converted into honey containing a pesticide till colony extinction or July 20th in the case that a colony survived after the administration of a pesticide was stopped on April 4th 2015. Strictly speaking, this assumption has few ambiguous points as follows: Honeybees in a colony may have ingested all of the toxic sugar syrup converted into honey and bee bead before the end of the experiment, or they do not always continuously ingest toxic honey and toxic bee bread stored in cells constantly through all seasons whether flowers are scarce or in full bloom.

With the aforementioned assumption, we estimated the intake of pesticide per bee. Accuracy in the total number of adult bees is deeply involved in accuracy in the intake of pesticide per bee which should be evaluated in consideration of the characteristics, especially, persistency of a pesticide, the full-bloom seasons and the behaviour and habits of honeybees. In this work, the problem as to how to deal with the determination of the duration to estimate the total number of adult bees in a bee colony which survives after the end of the experiment will remain. We determined a period of time from the start of experiment to the end of experiment as the duration to estimate the total number of adult bees when a colony survives at the end of the experiment, though we were worried as regards the selection of the period of time from the start of experiment till the finish of pesticide administration, or till a predetermined period of time after the finish of pesticide administration (for example, two or three months after the finish of pesticide administratin) when all the toxic honey and toxic bee bread stored in cells will be ingested after the discontinuation of pesticide administration, or till an appropriate time in the scarce-bloom season when honeybees begin to ingest honey stored in cells because honeybees prefer nectar from flowers to sugar syrup converted into honey stored.

Evaluation of the difference in insecticidal activity among pesticides

In order to compare the impact on a bee colony among pesticides, it is necessary to prepare the concentration of each pesticide so as to have the same insecticidal activity to honeybees. In this work we prepared the concentration of each pesticide relative to the insecticidal activity to exterminate stinkbugs as shown in Yamada et al. (2012), where the relative insecticidal activities to stinkbugs to clothianidin is 0.4 in dinotefuran and 0.08 in fenitrothion.

Generally, honeybees ingest sugar syrup converted into honey stored in cells during the scarce nectar-flow seasons when there are few flowers in bloom. On the contrary, honeybees preferentially ingest nectar and store most of sugar syrup while converting it into honey during the abundant nectar-flow seasons when flowers are in full bloom because honeybees prefer nectar to sugar syrup. As a pesticide is not effective for honeybees during the period when toxic sugar syrup is apparently consumed and stored in cells by honeybees, the time when honeybees consume toxic sugar syrup does not always coincide with the time when the impact of a pesticide on a bee colony appears. In that case there is a difference in the appearance time and strength of the impact among different pesticides. For example, a short-persistent pesticide such as the organophosphate fenitrothion lost its effect during the storage of honey and can hardly affect a bee colony even though honey is highly toxic as of the point in time when being stored. On the other hand, the toxicity of a long-persistent pesticide such as the neonicotinoids dinotefuran and clothianidin can be kept toxic even during a long-term storage of honey stored in cells on combs in a hive and can continue to adversely affect a bee colony for a long-term period of time.

Furthermore, we discussed the evaluation of the difference in insecticidal activity among pesticides. Firstly, examining the neonicotinoid dinotefuran colony group, DF-1 became extinct earlier on February 18th 2015 at the minimum intake of dinotefuran (0.2754 mg/colony, 32.95 ng/bee) among the dinotefuran colony group as shown in Tables 9 and 10. We deduced the reason for the early extinction at the minimum intake of dinotefuran of DF-1. As DF-1 which has fewer adult bees than the other colonies has fewer foraging bees than the others and as a result foraging bees in DF-1 can take less non-toxic nectar gathered from organically-grown flowers around the experimental site than the others; toxic sugar syrup containing dinotefuran fed into DF-1 cannot be sufficiently diluted in a cell with non-toxic nectar after toxic sugar is converted into toxic honey. Therefore, a certain amount of insufficiently-diluted toxic sugar syrup seems to have been stored as toxic honey and toxic bee bread in cells on combs in a hive after the start of dinotefuran administration on October 23rd 2014 shortly after the full-bloom season (Figure 5). When the scarce-bloom season sets in from the latter of November to the former half of February as shown in Figure 5, comparatively high toxic honey and toxic bee bread stored begins to be actively taken by honeybees and thereafter, adversely affected honeybees, a queen and brood, thereby resulting in a decrease in number of the adult bees and capped brood and finally DF-1 becomes extinct after DF-1 becomes so weak as to be unable to recover (Tables 7 and 8). DF-2 becomes extinct by similar reasons to DF-1. On the other hand, DF-3 takes abundant nectar from pesticide-free fields in October shortly after the full-bloom season, partly ingests it directly and stores it partly as thinner toxic honey and toxic bee bread in cells after being mixed with toxic sugar syrup because DF-3 has many adult bees and therefore seems to have enough foraging bees (Tables 7).

Abundant nectar in October in the bloom season makes DF-3 larger and thinner toxic honey and toxic bee bread stored in cells hardly make it become smaller in the scarce-bloom season from the latter half of November to the former half of February. After that, DF-3 becomes larger and more active in the full-bloom season from the former half of March to the former half of May because it takes non-toxic nectar rather than toxic honey stored in cells. In the full-bloom season from the former half of March to the former half of May, toxic sugar syrup fed to DF-3 is again stored in cells on combs in a hive as toxic honey or bee bread after being mixed with non-toxic honey. Once the scarce-bloom season has set in from the end of May to the end of July, DF-3 begins to take toxic honey and/or bee bread stored are weakened and finally become extinct around the end of the scarce-bloom season by the toxicity in honey and bee bread ingested and by the accumulated toxicity in a honeybee body by previous intakes of dinotefuran because the neonicotinoid dinotefuran is much longer persistent. This will be one of the feasible reports on the extinction of dinotefuran colonies.

Secondly, examining the neonicotinoid clothianidin colony group, CN-1 becomes extinct similar to DF-3 because adult bees and capped brood in CN-1 behaves in number analogously to those in DF-3 as shown in Tables 7 and 8 and Figures 4 and 6. The numbers of adult bees and brood in CN-1 and CN-2 comparatively analogously change with time except that CN-2 sharply decreases in the number of capped brood with time in the latter of October shortly after the start of pesticide administration. The difference in the number of capped brood between CN-1 and CN-2 was caused by the following mechanism: Toxic sugar syrup containing clothianidin taken by CN-2, whose colony size is smallest of all the clothianidin colony group, is converted into toxic honey and partly stored as toxic honey and toxic bee bread in cells in late October, nectar-flow season, while being mixed with non-toxic nectar. The toxicity of toxic honey and toxic bee bread in CN-2 seems to be higher than that in CN-1 because CN-2 is smaller in colony size than CN-1 and foraging bees in CN-2 also seems to be smaller in number than CN-1. Toxic bee bread may be fed to brood and a queen in CN-2 and as a result the brood may die and a queen may gradually decline in ovipositional capacity. Thereafter, CN-1 and CN-2 seem to have become finally extinct on July 20th 2015 by similar mechanism to DF-3 through the full-bloom season and the subsequent scarce-bloom season. On the other hand, judging from the fact that CN-3 begins to decrease in the numbers of capped brood from the end of January shortly before the full-bloom season and adult bees from the middle of February, some problems such as a functional disorder of oviposition of a queen and accidental deaths of brood due to the intake of pesticide (clothianidin) or a congenital disorder of a queen seems to arise in a queen or brood. We cannot apply the mechanism of CN-2 earlier mentioned to the accidental deaths of brood due to the intake of clothianidin in CN-3 because the colony size of CN-3 is largest of all in the clothianidin colony group. One of most feasible causes of the CN-3 extinction seems to be the abrupt onset of a congenital disorder of a queen. Another feasible cause considered is CN-3 whose colony size is largest among the clothianidin colonies and may have a certain amount of non-toxic honey and non-toxic bee bread stored in cells at the start of experiment; CN-3 will ingest the non-toxic honey and non-toxic bee bread during the scarce bloom season from the latter half of November to the former half of February. By the latter half of January,

CN-3 ingests up all the non-toxic honey and non-toxic bee bread stored. CN-3 ingests mainly non-toxic nectar and bee bread from pesticide-free fields during the bloom season from the start of experiment to the latter half of November when most toxic sugar syrup fed into CN-3 is converted into toxic honey and is stored as toxic honey and toxic bee bread in cells on combs in a hive while it is often mixed with non-toxic honey from organically-grown fields. After the latter half of January when all the non-toxic honey and non-toxic bee bread are ingested, CN-3 begins to ingest toxic honey and toxic bee bread stored in cells during the last bloom season. As a result CN-3 will be weakened by ingesting toxic honey and toxic bee bread and will finally become extinct.

Thirdly, examining the organophosphate fenitrothion colony group, we can understand that the short-term persistency in the organophosphate fenitrothion can strongly influence the extinction of colony only during a short period after the pesticide administration. All fenitrothion colonies (FT-1, FT-2 and FT-3) sharply decrease in the number of capped brood shortly after the start of pesticide (fenitrothion) administration but recover to about the original number after a month. Such a sharp decrease and rapid recovery of the number of capped brood in this work is similar to a sharp increase and rapid recovery of the number of dead bees in our previous work (Yamada et al., 2018b), where it has been deduced that such a change in the number of dead bee will be due to the short-term persistency of fenitrothion. The strange change in this work will occur as follows: The fenitrothion colonies will ingest partly toxic sugar syrup containing fenitrothion soon after fenitrothion is administered into a hive and they will store partly toxic sugar syrup as toxic food (toxic honey and toxic bee bread) in cells on combs in a hive after they mix toxic sugar syrup with non-toxic nectar and non-toxic pollen obtained from organically-brown fields. When toxic food is fed to brood soon after the start of administration of fenitrothion, it weakens brood fatally or causes brood to die but toxic food is rapidly weakened in toxicity and becomes non-toxic for a short period of time due to the short-term persistency of fenitrothion. The short-term persistency seems to make capped brood sharply decrease in number and rapidly recover.

Here, we focused on FT-1 which is the only extinct colony among the fenitorothion colonies. FT-1 sharply decreased in capped brood in the latter half of January again. The following reasons such as an ordinary decrease in oviposition due to the scarce bloom season, a death and injury of brood due to fenitrothion and an anomalous oviposition of a queen due to fenitrothion can be considered. It will be difficult to consider that the ordinary decrease in oviposition due to the scarce bloom season resulted in the sharp decrease in capped brood in January, because the sharp decrease is too great to consider being the ordinary decrease. It may be considered possible but ts possibility is not much high that the death and injury of brood due to fenitrothion causes sharp decrease in capped brood, because fenitrothion with short-term persistency seems to become less effective after about three month from the start of its administration. It will be a plausible and reasonable scenario that a queen which has ingested fenitrothion soon after the start of administration later begins to decrease in oviposition due to the toxicity of fenitrothion and capped brood decreases in number after lagging at least 21 days behind the oviposition. That is, fenitrothion seems to have been ingested by a queen around the latter half of December (the scarce bloom season) mainly through bee bread stored in cells or partly bee bread made of both toxic sugar syrup administered and pollen from fields. Two sharp decreases in the number of capped brood weakens FT-1; thereafter, FT-1 weakened

Table 12: Case study of the intake of pesticide per bees in FT-2 and FT-3 as a example of case where colony is surviving at the finish of experiment.

Name of colony	Kind of pesticide (concentration)	Duration of pesticide administration		Number of days in pesticide administration [days]	Estimated range which is under the influence of pesticide from the start of pesticide administration (October 23rd 2014)	Number of days in a estimated range which is under the influence of pesticide [days]	Total number of adult bees during the period between October 23rd 2014 and a given day (extinction and end of experiment., case study)	Revised total intake of pesticide per colony equivalent to clothianidiin [mg]	Revised intake of pesticide per bee equivalent to clothianidin [ng/bee]	State of colony at the end of experiment
DF-1	Dinotefuran (0.2 ppm)	From the start of pesticide administration (October 23rd 2014) to the colony extinction or to the stop of pesticide administration (April 4th 2015) on which a colony was surviving	This work	118	To the colony extinction	118	8359.3	0.1102	13.18	Extinct
DF-2				163		163	13001.2	0.9524	73.26	
DF-3				163		270	79370.7	3.5939	45.28	
CN-1	Clothianidin (0.08 ppm)			163		270	61267.9	2.097	34.23	
CN-2				163		222	28253.8	1.4825	52.47	
CN-3				141		141	18491.2	1.4058	76.03	
FT-1	Fenitrothion (1.0 ppm)			163		163	20039.8	2.1744	108.5	
FT-2				163	To the finish of experiment (July 20th in 2015)	270	82594.1	2.2464	27.20	Active
FT-3							108276.5	3.707	34.24	
FT-2			Case study	163	To the stop of pesticide administration (April 4th 2015)	163	32689.9	2.2464	68.72	
FT-3							52626.5	3.707	70.44	
FT-2				163	To 3 months later after the stop of administration (July 3rd 2015)	253	72613.3	2.2464	30.94	

begins to be attacked by vermin such as waxmoths, wax-moth larvae and hive beetles though fenitrothin ingested by FT-1 cannot lead to extinction and attacks by wax-worm larvae etc (Table 5) will make FT-1 escape from its hive which is uncomfortable.

FT-2 and FT-3 decreased in the number of capped brood shortly after the start of administration of fenitrothion; they soon recovered to about the original state. Thereafter, they survived to the end of the experiment while staying active though they continue to ingest fenitrothion through toxic honey stored in cells on combs whose toxicity becomes rapidly weaker with the lapse of time. The reason why FT-2 and FT-3 was able to survive was because fenitrothion which is readily degradable loses its effectiveness as the time proceeds. FT-3 was most active of all the colony groups.

Evaluation of the intake of pesticide per bee in a surviving colony

It is very difficult to determine a time period over which honeybees ingest a pesticide whose effectiveness holds long in a field experiment because honeybees have such a habit as storage of food in cells on combs and preference of natural food over its substitutes. For examples, when an administration period of pesticide is determined as a honeybee-ingesting period, the honeybee-ingesting period may be underestimated by neglecting a period of time when honeybees ingest food stored in cells after the discontinuation of pesticide administration. In this case, the total number of adult bees will also be underestimated than the actual number because a period of time to estimate the total number of adult bees is shortened and thus the intake of pesticide per bee obtained will be estimated more than the actual number. On the other hand, when a longer honeybee-ingesting period than the actual honeybee-ingesting period is adopted, the honeybee-ingesting period will be overestimated and the total number of adult bees will also be overestimated than the actual number and thus the intake of pesticide per bee obtained will be estimated lower than the actual intake of pesticide per bee.

There is another difficult problem to be solved on the honeybee-ingesting period among pesticides having different characteristics such as different persistency and repellency or among colonies with a queen having different characteristics from each other. For example, when pesticides with long persistency and those with short persistency are in a field experiment such as this work, we worry about how to determine the honeybee-ingesting period, whether we should alter it considering the characteristic of each pesticide or fix it at a given period such as a pesticide administration period independently of the characteristics of pesticide such as this work. We examined the influence of the honeybee-ingesting period on the intake of pesticide per bee about three cases of a period of pesticide administration, a period between the start of pesticide administration to the end, a period between the start of experiment to the end and a period from the start of experiment to three months after the stop of pesticide administration where all toxic food stored in a hive will be ingested by honeybees. Table 12 shows examples of the total number of adult bees of FT-2 and FT-3 in each period and the intake of pesticide per bee equivalent to clothianidin. It is evident from Table 12 that both the total number of adult bees and the intake of pesticide per bee strongly depend on the honeybee-ingesting period. In this work, we adopted the period between the start of experiment and the end as the honeybee-ingesting period. Comparing the intake of fenitrothion per bee in FT-2 and FT-3 among three periods, it became lower in order of the period of fenitrothion administration (163 days), the period from the start of pesticide administration to three months after the stop of fenitrothion administration (253 days) and the period between the start of pesticide administration to the end of the experiment (270 days) in both FT-2 and FT-3. For example, the intake of fenitrothion per bee in the fenitrothion administration period is more than twice of that in the period between the start of experiment to the end. As the intake of pesticide per bee depends on a honeybee-ingesting period, it is desirable to carefully determine a plausible honeybee-ingesting period under the available information after examining it from various angles such as a bloom season, an amount of honey and bee bead stored in cells on combs and the characteristics of pesticide. It can been seen from three case studies on this work in Table 12 that FT-2 and FT-3 which have survived to the end of the experiment ingest comparable or more fenitrothion in comparison with all the neonicotinoid colonies, which have become extinct during the experimental period.

Further, an additional problem is that not all honeybees ingest the same amount of pesticide during an experiment period. When there is the inequality of the pesticide intake in a colony among experimental colonies, a difference in colony activity may occur among them even if mean intake of pesticide per bee is the same. The reason is, for example, that the experimental results where all house bees have ingested a lethal dose of pesticide seem to be different from those where foraging bees have ingested. It will be almost impossible to solve the aforementioned problem in a long-term field experiment because it is necessary to count the dead bees while confirming a role played in a honeybee colony one after the other. In order to solve such various problem, usually, the results obtained from many colonies are statistically analysed. An experiment with many colonies whose number makes a statistical analysis possible seems to be impossible in a long-term field experiment. Therefore, it will be necessary to conduct a long-term field experiment as accurately and replicable as possible using the minimum number of colonies.

Conclusion

The field experiment with twelve colonies was conducted in order to examine the long-term impact of the neonicotinoids dinotefuran and clothianidin and the organophosphate fenitrothionon honey bee colony in about 220-acre organically-grown macadamia trees in seasonless and mite-free Maui from late October, 2014 to late July, 2015. A concentration in sugar syrup containing each pesticide was administered into three colonies is one-five hundredth of a recommended concentration to exterminate stinkbugs in the farmland. From the long-term field experiment, we can deduce that neonicotinoid pesticides exterminate honeybee colonies with much higher probability than organophosphate pesticides which have the same extinction probability as control colonies. This finding obtained in Maui where there is no mite harmful to honeybees is almost the same as the finding obtained in Japan where there are mites (Yamada et al., 2012; 2018b, c). Organophosphate fenitorothion colony where fenitrothion was administered became extinct not due to the toxicity of fenitorothion but due to the escape from the hive as a result of attacks by harmful insects such as wax-moth larvae as in the case of the control colony which became extinct.

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