Neonicotinoids and *Varroa* mites force the extending of longevity in a bee colony during overwintering to forget

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Abstract

A mathematical model that can estimate the apparent longevity of bee-colony proposed by the authors to clarify that the longevity normally changes with seasons as follows: The longevity maintains almost constant from at 20 to 30 days from late spring to late autumn, thereafter, it continues to extend till late spring up to 160 to 200 days. Immediately after overwintering, the longevity is shortened almost vertically from one sixth to one tenth. Such normal seasonal changes in longevity are shown when a pesticide-free food is administered to the bee-colony and when pesticide-containing sugar syrup is. However, abnormal seasonal changes in longevity, which does not extend even if winter approaches, are shown for the bee-colony ingesting neonicotinoid-containing pollen and for the colony infested with *Varroa* mites.

Judging from the fact that pollen is the main food for the bee-brood, that mites parasitize larvae and pupae, and that the vital functions and organs of honeybees are created during the larval and pupal stages, it can be inferred that a neonicotinoid-containing pollen paste and parasitic mites cause serious damage to the bee ability to detect the arrival of winter. Such dysfunction in the larval and pupal stages probably interferes with extending the longevity of adult bees even as winter approaches.

Key words: seasonal changes, longevity, bee colony, overwintering, sugar-syrup pollen-paste, neonicotinoid, *Varroa* mite

Introduction

Lifespan of animal individuals is one of the important indicators for evaluating the activity of animals. In eusocial insects that form colonies such as honeybees, colonies can be treated as super-individuals that behave as if they were a single animal. It can be said that the day age (longevity) of the longest-living group of honeybees in a honeybee-colony is representative of the period of activity of the honeybee-colony. In other words, the activity period of a honeybee-colony is considered to be assessed by longevity which is an indicator of the life of the entire colony, rather than the lifespan of an individual bee.

The authors proposed a mathematical model (Y. Yamada et al., 2019) that estimates the longevity of the honeybee-colony only from the number of adult bees and the number of capped brood, and named the longevity estimated by the mathematical model "apparent longevity" in order to distinguish it from that measured directly. Using this proposed mathematical model and the number of adult bees and the number of capped brood measured in long-term field-experiments, we estimated seasonal changes of apparent longevity (Y. Yamada et al., 2019).

It was found that the apparent longevity of control (pesticide-free) colony estimated using long-term field-experiments in mid-west Japan extended rapidly during wintering, and that just before the end of wintering, the apparent longevity reached 6 to 10 times longer than that in non-winter seasons (Y. Yamada et al., 2019). Such a sudden change in apparent longevity cannot be explained only by the labor loads (Fukuda &Sekiguchi, 1966) such as foraging tasks (Schmid-Hempel &Wolf, 1988; Wolf & Schmid-Hempel, 1989) and nursing tasks (Amdam et al., 2009) that have been said so far. Therefore, the authors have proposed a hypothesis that a biological rhythm control system which is genetically pre-incorporated in a honeybee detects seasonal changes in the environment such as temperature, humidity and sunshine duration and results in causing seasonal changes in longevity (Y. Yamada et al., 2019).

In addition, the apparent longevity estimated from long-term field-experiment results on Maui without cold winters was quite different from the estimated results in mid-west Japan, and significantly fluctuated, but no seasonal changes were observed. In less seasonal-change areas such as Maui, the apparent longevity has been found to be greatly affected by physiological phenomena in honeybees, such as the absence of queens and a decrease in egg production (T. Yamada & K. Yamada, 2020).

There have been many reports on factors affecting the longevity of honeybees. Neonicotinoid pesticides (neonicotinoids) that appeared at the end of the 20th century are known to adversely affect the longevity of honeybees due to their characteristics (long-persistent, highly-insecticidal, systemic, neurotoxic). For example, neonicotinoids themselves directly shorten the longevity of honeybees (Tarek et al., 2018; Anderson et al., 2019; Aljedani, 2017), or indirectly shorten their longevity as follows:

Neonicotinoids can adversely affect the immunity of queen bees and cause the re-development of the disease (Brandt et al., 2017); they disrupt the circadian rhythms and sleep of honeybees and can impair honeybee navigation, time memory and social communication (Tackenberg et al., 2020); acetamiprid adversely affects memory-related properties in honeybees, homing ability and expression

levels of memory-related genes (Shi et al., 2019). Neonicotinoids reduce the mating frequency of queens, resulting in a decrease in the genetic diversity of working bees, which can adversely affect colony vitality (Forfert et al., 2017); imidacloprid damages the development of the nervous system in areas responsible for both the sense of smell and vision in the larval stage of honeybees, inhibiting the activity of honeybees (Peng & Yang, 2016).

Moreover, *Varroa* mites also shorten the longevity of honeybees as follows: *Varroa* mites and deformed wing virus (DWV) reduce the lifespan of winter honeybees (Dainat et al., 2012); adult bees emerging from capped brood in the presence of *Varroa* mites cause their deformed wing and significantly reduce their longevity (Martin, 2013); external parasitic mites, *Tropilaelaps mercedesae*, reduce the longevity and emergence weight of honeybees (Khongphinitbunjong, 2016); *Varroa* destructor parasitic disease and DWV infections cause deformities during the development of Africanized bees, inhibiting cellular immunity and significantly reducing the lifespan of adult bees (Reyes-Quintana et al., 2019).

In addition, combination of the chronic exposure to the systemic pesticide (neonicotinoids and fipronil) and the infestation of the *Varroa* mite (Straub et al., 2019) or the microsporidian parasite Nosema ceranae which is deeply involved with the *Varroa* mite (Alaux et al. 2010; Vidau et al, 2011; Aufauvre et al., 2012; Aufauvre et al., 2014; Gregore et al. 2016) has synergistic and adverse impact on honeybee survival, and results in shortening the longevity of honeybee-colony.

Thus, while it has been found that the long-persistent and systemic pesticide (*e.g.* neonicotinoids) and the external parasitic mite (*e.g. Varroa* mites) and/or viruses mediated by those mites reduce the longevity of honeybees, we cannot find papers regarding the impacts of systemic pesticides and/or *Varroa* mites on seasonal changes in honeybee longevity other than in our study (T. Yamada & K. Yamada, 2020).

Therefore, since it is important to know what gives to seasonal changes in the longevity of bee colonies in beekeeping, this paper clarifies the following points using a mathematical model (Y. Yamada et al., 2019) that estimates the longevity of honeybee *Apis mellifera* colonies from only the numbers of adult bees and capped brood obtained from long-term field-experiments (T. Yamada et al., 2018a; 2018b; 2018c) conducted in the mid-west of Japan: **1)** Under the *Varroa* mite-free circumstances, on the difference in the seasonal changes in apparent longevity among the three kind of colonies, without pesticides (control) (hereafter, mite-free-CR), with the neonicotinoids dinotefuran (hereafter, mite-free-DF/SS) and clothianidin via sugar syrup (hereafter, mite-free-CN/SS) and with the organophosphates fenitrothion and malathion via sugar syrup (hereafter, mite-free-FT/SS and mite-free-MT/SS): **2)**On the difference in the seasonal changes in apparent longevity between two kinds of vehicles, sugar syrup (hereafter, SS) and pollen paste (hereafter, PP), to administer a pesticide: **3)**On the difference in the seasonal changes in apparent longevity between the mite-free-CR colony and the colony where no pesticide is administered and mites are infested (hereafter, mite-infested CR colony).

Materials and Methods

For details, please refer to Methods S1-S3 in the supplementary materials.

Determination method of apparent longevity (refer to Supplementary Method S1)

【Mathematical Model】

The authors have proposed a mathematical model that can estimate the apparent longevity from the numbers of adult bees and capped brood (Y. Yamada et al., 2019). Here a dynamic parameter called "apparent longevity", *L (t)*, defined in Equation (S1) is introduced, which depicts the characteristics of the "boundary" day-age where the average honeybee-worker disappears from the bee colony:

$$a(t) = \int_{T_{pupa}}^{T_{pupa}+L(t)} u(t-s)p(t-s)ds \qquad \text{(S1)},$$

where *a(t)* and *u(t)* are the number of adult bees and the number of newly capped brood per unit time *t*, respectively. *p (t)* is the eclosion rate from capped brood (pupae in comb-cells with a sealed entrance) at time *t*. T_{pupa} is the average period of capped brood, and the period of honeybees *Apis mellifera* is 12 days. If the bee colony is in a steady state, the worker bees die at the same age. *L(t)* represents the longevity of an adult bee. The age distribution of dying bees is given by the narrow Gaussian distribution.

Therefore, it is worth noting that Equation (S1) may provide an estimate of the longevity of a worker bee in a normal bee group.

In order to find the apparent longevity, *L(t)*, in Equation (S1), it is necessary to know *a(t)*, *u(t)* and *p(t)*. The values of *a(t)* and *u(t)* will be obtained from measurements in field experiments. Since it is difficult to directly measure *u(t)*, it is estimated from Equation (S2) using the *b(t)* value measured by the field experiment.

$$b(t) = \int_{0}^{T_{pupa}} u(t-s)[p(t-s)]^{s/T_{pupa}} ds \qquad \text{(S2)},$$

where *b (t)* is the number of capped brood at time *t*. Equation (S2) assumes that the pupa is removed as soon as it is dead in the comb cells.

By combining Equations (S1) and (S2), it is possible to characterize the honeybee group using a macroscopic amount on honeybees (the number of adult bees and number of capped brood) that are easy to get by experiments. Although *a(t)* in Equation (S1) and *b(t)* in Equation (S2) are continuous in the formula (it is a continuous function), it is actually impossible to measure them continuously in field experiments. Since the measurement date is discrete, Equations (S1) and (S2) were separated under the following assumptions.

1) The day-age distribution of capped brood on a measurement date is assumed to be discretely uniform during the capping period on the comb-cells (12 days), and to be constant between two consecutive measurement dates (in each measuring interval). These assumptions are equivalent to that the number of newly capped brood per day, *u(t)*, is constant between two consecutive measurement dates. If the number of capped brood measured on a measurement date is expressed in $b(t_k)$, the daily average number of adult bees that newly emerges from the capped brood between one measurement date and the next measurement date is given by $b(t_k)/T_{pupa}$.

2) The eclosion rate is assumed to be constant in two consecutive measurement dates.
3) It is assumed that the pesticide intake per larva in two consecutive measurement dates is assumed to be the same as the intake of pesticides per bee. In our experiments, it is not possible to directly measure the intake of pesticides per larva between two consecutive measurement dates. Therefore, the pesticide intake per of the larva is substituted for that the that per measurable adult bee.
In Equations (S1) and (S2), *a(t)* and *b(t)* are known values measured in field experiments, but *L(t)*, *u(t)* and *p(t)* are unknown. In two simultaneous equations, one of these three unknown variables must be known before these simultaneous equations can be solved. Fortunately, the eclosion rates, *p(t)*, at various imidacloprid doses has already been reported (Yang et al. 2012). The eclosion rate required for calculating the apparent longevity can be obtained using the mean eclosion rate curve in Supplementary Figure S1.
Since the pesticide used in this experiment is dinotefuran, not imidacloprid, the dinotefuran concentration must be converted to the imidacloprid dose in order to use Supplementary Figure S1. Therefore, the imidacloprid dose equivalent to the insecticidal ability of a certain dinotefuran concentration is estimated as follows.
Field experiment conducted by the authors (T. Yamada et al., 2012) shows that the insecticidal ability of clothianidin for honeybees is about 2.5 times that of dinotefuran. It can be assumed that imidacloprid's ability to exterminate honeybees is about 2.5 times that of dinotefuran, since the recommended insecticidal concentration of imidacloprid for exterminating stink bugs is approximately the same as that of clothianidin.
Pesticide intake per bee in two consecutive measurement dates can be estimated from the total pesticide intake and total number of adult bees between the measurement dates using the method proposed by the authors (T. Yamada et al., 2018a).
In the range of pesticide dosages in previous field experiments (see Supplementary Table S1), as shown in Supplementary Figure S2, the effect on the eclosion rate from bee pupae, *p(t)*, of the pesticide dose is small. That is, it can be assumed that the eclosion rate is equal and constant to 0.9 of the control group. Therefore, in this paper, the apparent longevity was calculated with a constant eclosion rate of 0.9 throughout the experiment as previously reported (T. Yamada & K. Yamada, 2020). Using the dataset at each measurement date obtained from the field experiment, the apparent longevity, *L(t)*, can be obtained by solving the discrete equation as shown in the previous report (Y. Yamada et al., 2019).

【Determination Procedure of Apparent Longevity】

The unknown variables in the equations for determining the apparent longevity of a bee colony in each measurement (observational experiment) are the apparent longevity *L(t)* and the number of the newly capped brood *u(t)*. Of these two variables, *u(t)* can be obtained from Equation (S2). Of these two variables, u(t) can be determined from Equation (S2).
Therefore, the *u(t)* value determined from Equation (S2) is substituted to Equation (S1), and the unknown variable *L(t)* in Equation (S1) is determined by an iteration method such as the Bisection

method (Solanki et al., 2014) and the Simplex method (Nagata & Yamada, 1973). While changing the value *L(t)*, a certain value *L(t)* is repeatedly substituted for Equation (S1). And then, the calculations of discretization equations for Equations (S1) and (S2) are repeated until a difference between the calculated value of the number of adult bees a(t) obtained by the iteration method and the measured value of the number of adult bees by a field experiment reaches a given convergence criterion value (0.0001 in this paper) or less.

In the above calculation to seek the apparent longevity *L (t)*, actual measurement data can be obtained only for each measurement date. Therefore, the apparent longevity *L(t)* can be sought after the discretization of the equations while taking account of the life cycle from oviposition to eclosion of the honeybee (egg period 3 days; larval period 6 days; pupa (capped brood) period 12 days).

In this study, the apparent longevity *L(t)* is determined by the Bisection method (the convergence radius of L(t) is 0.0001 in this work). The calculations were performed using the Ruby programming language (Flanagan & Matsumoto, 2016). For more details such as the procedure and flowchart to seeking an apparent longevity, refer to Supplementary Method 1.

Materials used in long-term field experiments

Neonicotinoid pesticides (dinotefuran, clothianidin) and organophosphate pesticides (fenitrothion, malathion) were used in long-term field experiments (T. Yamada et al., 2012; 2018a; 2018b; 2018c; 2018d; T. Yamada, 2020) as follows: Dinotefuran (neonicotinoid pesticide) (DF): Starkle Mate® (10% DF; Mitsui Chemicals Aglo, Inc., Tokyo, Japan). Clothianidin (neonicotinoid pesticide) (CN): DANTOTSU® (16% clothianidin; Sumitomo Co. Ltd., Tokyo, Japan). Fenitrothion (organophosphate pesticide) (FT): SUMITHION® emulsion (50% fenitrothion; Sumitomo Co. Ltd., Japan) was used. Malathion (organophosphate pesticide) (MT): MALATHON® emulsion (50% malathion; Sumitomo Co. Ltd., Japan).

Sugar syrup (SS) and pollen paste (PP) are used not only as food for bees, are often used but also as a vehicle for experiments in which pesticides are administered to bee colonies.

In this study, SS consists of 60 % sugar by weight and 40% water by weight, and was made by dissolving sugar in water at 70 °C. Sugar is granulated sugar that was composed of 99.7988% purified sugar (granulated sugar), Sodium chloride (salt) 0.1% or more, L-lysine hydrochloride 0.1% or more, and food dye (Blue No. 2) 0.0012% or more), and was purchased from the Japan Beekeeping Association (http://www.beekeeping.or.jp/). PP consists of 60% pollen by weight and 40% SS by weight, and was kneaded with a high-viscosity stirring bladed drill driver to a homogeneous paste at low speed while adding pollen to the SS.

For SS containing a pesticide, the desired amount of the pesticide was mixed into pesticide-free SS so that the desired concentration was achieved. PP containing a pesticide was made by kneading pollen into SS containing the desired pesticide concentration.

More details are described in Supplementary Method S2.

Preparation for field experiment

In field experiments to investigate the effects of pesticides on bee colonies, it is necessary to minimize

the effects of pesticides other than the pesticides administered in the experiments. Therefore, we tried to make the area around the experimental site as pesticide-free as possible. In other words, in the long-term field experiments in the Midwest of Japan, a water-feeding spot and foraging ground for bees were built in the immediate vicinity of the experimental site. Specifically, a system was installed that automatically supplies fresh water from a large tank. And, in a pesticide-free environment, pesticide-free plants and fruit-bearing trees were planted and cultivated. More details are described in Supplementary Method S2.

Procedure for field experiment

The most important data in the field experiment of honeybee colony are both the number of adult bees and capped brood, which are indicators of the size, strength, vitality and power of the colony. To obtain these data as accurately as possible, the field experiment was carried out. The detailed procedure is described in Supplementary Method S2.

Counting methods of adult bees, capped brood, mite-damaged bees

Using the photographs taken in field experiments, it was possible to determine the numbers of adult bees, capped brood and mite-damaged bees. Counting such numbers from photographs is an extremely difficult task. Therefore, with the cooperation of Mr. Yoshiki Nagai of Nanosystem Co., Ltd., Kyoto, Japan (http://nanosystem.jp/firm.htm), which develops image processing software, the author has developed computer software in 2012 that automatically counts the relevant numbers from photographic images, and improvements have been made to the software since. The accuracy and operation of the counting have been greatly improved. By using this software, the time and accuracy for counting such numbers have been significantly improved, compared to the amount of work required to count by hand directly from the original photo.

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

When counting the number of *Varroa* mites, it is impossible to count *Varroa* mites that causes damage to a brood in a comb cell. If any damage is received from *Varroa* mites during the brood stage, there should be a trace of the damage even if the brood becomes an adult bee. Therefore, assuming that such traces confirm the presence of mites, we checked the adult bees for traces of mites. Based on the criteria for determining the traces due to *Varroa* mites (Supplementary Figure S5), the number of the traces (including *Varroa* mite itself) is counted on the photographic image while being enlarged with the help of the automatic counting system newly developed. More details on counting methods of the numbers of adult bees, capped brood and mite-damaged bees are described in Supplementary Method

S3.

Results

Here, we will examine the following seasonal changes in apparent longevity of colonies, which was estimated though a mathematical model (Y. Yamada et al., 2019) using the numbers of adult bees and capped brood measured in long-term field experiments (T. Yamada et al., 2018a; 2018b; 2018c; 2020).

Mite-free-CR colony

The mite-free-CR colony (2011/2012 CR-1; 2012/2013 CR-1; 2012/2013 CR-2; 2013/2014 CR-1; 2013/2014 CR-2) (T. Yamada et al., 2018a; 2018b; 22018c) shows seasonal changes in apparent longevity that begins to extend around late September, becomes the longest (about 160 to 200 days) just before the end of overwintering, becomes instantly short up to near low levels (20 to 30 days) just after overwintering, and then stays almost constant at low levels, albeit with small fluctuations (Figure 1A; Supplementary Figures S7 to S9) (Y. Yamada et al., 2019). Such seasonal changes in apparent longevity are normal in areas with four seasons until the colony extinction.

Such normal changes in apparent longevity that extends as winter approaches cannot be always explained by the conventional theories such as workload on honeybees (Schmid-Hempel &Wolf, 1988; Amdam et al., 2009; Omholt, 1988) and nutritional intake (Alqami, 2006; Wang et al., 2014; Yang et al., 2017); for example, the facts that the apparent longevity begins to rise around late September when honeybees are active; that the apparent longevity continues to extend during the overwintering season when bees don't always have enough food and even when a queen bee begins to oviposit in mid-February, and so on. The extending of apparent longevity as winter approaches can be caused by the genes already embedded so as such facts are steered.

Mite-free-neonicotinoids/SS (mite-free-DF/SS and mite-free-CN/SS)

The mite-free-DF/SS colony (2011/2012 DF 10ppm/SS; 2013/2014 DF 0.2ppm/SS) (T. Yamada et al., 2018a; 2018c) and the mite-free-CN/SS colony (2013/2014 CN 0.08ppm/SS) (T. Yamada et al., 2018c) show seasonal changes in apparent longevity that begins to extend around late September as in case of the mite-free-CR colony but decrease rapidly soon after the beginning of the overwintering because of the colony extinction (Figure 1A; Figures S7 & S9). The colony extinction can be inferred to be caused by the weakening of the colonies due to the long-persistent toxicity of dinotefuran or clothianidin.

Mite-free-organophosphates/SS (mite-free-FT/SS and mite-free-MT/SS)

The mite-free-FT/SS colony (2012/2013 FT 10ppm/SS) (T. Yamada et al., 2018b) exhibits normal seasonal changes in apparent longevity and success in overwintering almost the same as in case of the mite-free-CR colony (Figure 1A; Supplementary Figure S8). On the other hand, The mite-free-FT/SS colony (2013/2014 FT 1ppm/SS) (T. Yamada et al., 2018c) and the mite-free-MT/SS colony (T. Yamada et al., 2018c) exhibit seasonal changes in apparent longevity that begins to extend around late September as in case of the mite-free-CR colony but reduce sharply in the middle of the

overwintering because of the colony extinction (Figure 1A; Supplementary Figure S9). The colony extinction can be inferred to be caused by the slight-weakening of the colonies due to the short-term lasting toxicity of fenitrothion and malathion.

Mite-free-DF/PP

The mite-free DF/PP colony (2011/2012 DF 0.565/ppm) (T. Yamada et al., 2018a) shows abnormal seasonal changes in apparent longevity that does not begin to extend even late September and keeps approximately constant till the colony extinct (Figure 1B; Figure S7). The colony extinction can be inferred to be caused by the weakening of the colonies due to the long-persistent toxicity of dinotefuran.

Mite-infested-CR

The mite-infested-CR colony (2018 CR-1; 2018 CR-2; 2018 CR-3) (Yamada, 2020) also shows abnormal seasonal changes in apparent longevity. Far from beginning to begin to extend late September, the apparent longevity begins to decrease and the colonies become extinct after the start of overwintering (Figure 1B; Supplementary Figure S10).

Mite-infested-DF/PP

The mite-infested-DF/PP colony (2018 DF-2) (2020) also shows abnormal seasonal changes in apparent longevity in the same as in case of the mite-infested-CR; far from beginning to begin to extend late September, the apparent longevity begins to decrease and the colonies become extinct after the start of overwintering (Figure 1B; Supplementary Figure S10).

Discussion

Focusing on how abnormal changes in apparent longevity are caused, we will discuss various differences of seasonal changes in apparent longevity.

The difference in seasonal changes in apparent longevity among mite-free-CR, mite-free-DF/SS and mite-free-CN/SS, mite-free-FT/SS and mite-free-MT/SS

Until the colony extinction, the apparent longevity of both the mite-free colony where no pesticide is administered and a pesticide is administered via SS shows almost the same normal-changes of the seasons. When the colony is strong, it can succeed in overwintering, and when the colony is weakened by the pesticide or others, it will fail in overwintering (Figure 1A; Supplementary Figures S7 to S9). The reason why the pesticide administered to the colony via SS cannot cause abnormal changes in apparent longevity will be probably because SS (honey) is an energy source. Incidentally, genes, biological functions and organs are created in egg, larval and pupal stages of honeybees. In addition, SS (honey) is ingested for bees' activities mainly by adult bees. but not ingested almost by a queen bee, larvae and pupae. Therefore, SS can hardly cause abnormal changes in AL.

The difference in seasonal changes in apparent longevity between two kinds of vehicles (SS, PP) to administer a pesticide under the mite-free circumstances

The mite-free colony where a pesticide is administered via SS (2011/2012 DF 10ppm/SS; 2013/2014 DF 0.2ppm/SS; 2012/2013 FT 10 ppm/SS; 2013/2014 1ppm/SS; 2013/2014 MT 1ppm/SS) exhibits normal seasonal changes in apparent longevity that begins to extend from late September and continues to extend till the colony becomes extinct during overwintering. On the other hand, the mite-free colony where a pesticide is administered via PP (2011/2012 DF 0.565ppm/PP) exhibits abnormal seasonal changes in apparent longevity; the apparent longevity keep approximately constant in all seasons, even during overwintering, till the colony extinct as if the colony cannot discern the approach of winter (Figure 1B; Supplementary Figures S7 to S9).

The difference in the vehicle to administer a pesticide can cause the above difference in seasonal changes in apparent longevity.

Honey (SS) is the staple food of adult bees and pollen (PP) is the staple food of larvae and pupae. In addition. royal jellies which are made from pollen are ingested by queen bees and young larvae. Eggs are oviposited by a queen bee and functions and organs of honeybees are made in larval and pupal stages. From the above, pesticides involved in pollen can adversely affect honeybees during gene transcription, functional differentiation and organ creation. Long-persistent pesticides with high toxicity and neurotoxicity, such as neonicotinoids, will continue to adversely affect honeybees over a long period of time once they are ingested during the egg, larval and pupal stages. Such long-term adverse impact can cause gene transcription errors or some kind of dysfunction such as biological rhythm disorder. Such impediments will make it difficult to predict the approach of winter.

The difference in seasonal changes in apparent longevity between the mite-free-CR colony and the mite-infested CR colony

The mite-free-CR colony (2018 CR-1; 2018 CR-2; 2018 CR-3) exhibits normal seasonal changes in apparent longevity, while the mite-infested-CR colony exhibits abnormal seasonal changes in apparent longevity similar to the mite-free-DF/PP colony (2011/2012 DF0.565ppm/PP) (Figure 1B; Supplementary Figures S7 & S10).

It can be assumed that the difference between these seasonal changes in apparent longevity is due to the fact that *Varroa* mites attack larvae and pupae (Sammataro, et al., 2000), in whose stages functions and organs of honeybees are made, and cause damage them. It can be assumed that mites and vector-borne viruses in the larval and pupal stages disrupt or destroy the honeybee's biological clock and sensory organs, with making it impossible to recognize seasonal changes.

Though seasonal changes in apparent longevity of mite-infested colonies should hold constant in all seasons till the colony extinction in this case, the fact that they decrease from mid-September till the colony extinction can be caused by the extreme weakening of the colonies due to higher than 50% of mite-prevalence (Figure 2). In addition, there is little difference in seasonal changes in apparent longevity between the mite-infested CR colony and the mite-infested DF/PP colony. The reason will be that the influence of damages by mites is too strong.

Incidentally, it is suspected that the fact that seasonal changes in apparent longevity in mite-free

DF/PP colony may be due to mite-infection. Therefore, to confirm the infection status of mites, the number of damaged bees by mites in the mite-free-DF/PP colony was counted according to the sections 1.3.3 and 1.3.4 in Supplementary Method S3. As a result, it turned out to be that the mite-free-DF/PP colony is hardly infested with mites because its mite-prevalence is 5% or less, while that of the mite-infested colony reaches over 90% (Figure 2). It was verified that the previous report (T. Yamada et al., 2018a) that there are no mites is correct, and the apparent longevity constant seasonal-change of the mite-free--DF/PP colony is not due to the infestation of mites, but to the ingestion of dinotefuran via PP.

Conclusions

The mite-free CR colony and the colony where a pesticide is administered via SS colony exhibit normal seasonal changes in apparent longevity that begin to extend from late September till the colony extinction, while the mite- infested colony and the mite-free DF/PP colony exhibit abnormal seasonal changes in apparent longevity that keep constant in all seasons or decrease even when winter approaching till the colony extinct. Such abnormal seasonal changes in apparent longevity are inferred to be caused by serious damages on eggs, larvae and pupae.

Supplementary material

Supplementary Methods S1 to S3; Supplementary Figures S1 to S10; Supplementary Tables S1 to S7.

Acknowledgments

We would like to greatly appreciate the hearty cooperation of Mr. Yuhki Nagai (Nanosystem Co., Ltd., Kyoto, Japan) for developing and improving automatic counting software to improve the counting speed and accuracy of the numbers of adult bees, capped brood and damaged mites. We would like to thank for the cooperation of Mr. Kazuo Harada (Platon Co., Ltd., Kakogawa, Japan) when improving the input and output of software with apparent longevity. We would also like to express their deep gratitude to the following people for their devoted cooperation in conducting the field-experiments and counting the numbers of adult bees and capped brood whose measurements proved to be highly accurate through the estimation of apparent longevity with a mathematical model; Ms. Kazuko Yamada; Ms. Hiroko Nakamura; the members of YUINOTE (Mr. Tetsuya Kojima, Ms. Yuki Kojima, Dr. Hiroshi Mibayashi, Ms. Kimiko Mimura-Ruth, and Mr. Kenji Mimura) which is a natural farming group in Noto, Japan. We appreciate also Ms. Kazuko Yamada's cooperation in algorithmic design for longevity estimation. Dr. Ken Hashimoto (Okayama, Japan) gave us his warm and appreciate advice, guidance and encouragement when we had some troubles on this research. Thank you deeply. Finally, we would like to thank everyone who gave us this opportunity and/or assisted us for our research.

Disclosure statement

No potential conflict of interest was reported by the authors.

Funding

There was no funding received for this work.

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References

References with an underscore for the first author (three references) are cited in the supplementary material, and the rest are cited in the text.

Alaux C., Brunet J. L., Dussaubat C., Mondet F., Tchamitchan S., Cousin M., Brillard J., Baldy A., Belzunces, L. P. & Le Conte, Y. (2010). Interactions between Nosema microspores and a neonicotinoid weaken honeybees (*Apis mellifera*). *Environmental* Microbiology, *12*(3), 774–782. https://doi.org/10.1111/j.1462-2920.2009.02123.x.

Aljedani, D. M. (2017). Detection of toxicity and effects of some insecticides to local honey bee race (*Apis mellifera jemenatica*). *Journal of the American Chemical Society*, *13*(3), 19–31. https://doi.org/10.7537/marsjas130317.04.

Alqami, A. S. (2006). Influence of Some Protein Diets on the Longevity and Some Physiological Conditions of Honeybee *Apis mellifera L.* Workers. *Journal of Biological* Sciences, *6*(4), 734–737. https://doi.org/10.3923/jbs.2006.734.737.

Amdam, G. V., Rueppell, O., Fondrk, M. K., Page, R. E., & Nelson, C. M. (2009). The nurse's load: Early-life exposure to brood-rearing affects behavior and lifespan in honey bees (*Apis mellifera*). *Experimental* Gerontology, *44*(6−7), 467–471. https://doi.org/10.1016/j.exger.2009.02.013.

Anderson, N. L., & Harmon-Threatt, A. N. (2019). Chronic contact with realistic soil concentrations of imidacloprid affects the mass, immature development speed, and adult longevity of solitary bees. *Scientific. Reports*, *9*, 3724. https://doi.org/10.1038/s41598-019-40031-9.

Aufauvre, J., Biron, D. G., Vidau, C., Fontbonne, R., Roudel, M., Diogon, M., Viguès, B., Belzunces, L. P., Delbac, F., & Blot, N. (2012). Parasite-insecticide interactions: a case study of Nosema ceranae and fipronil synergy on honeybee. *Scientific Reports*, *2*, 326. https://doi.org/10.1038/srep00326.

Aufauvre, J., Misme-Aucouturier, B., Vigues, B., Texier, C., Delbac, F., & Blot, N. (2014). Transcriptome analyses of the honeybee response to *Nosema ceranae* and insecticides. *PLoS ONE*, *9*(3), e91686. https://doi.org/10.1371/journal.pone.0091686.

Barlow V.M., & Fell, R.D. (2006). Sampling Methods for *Varroa* Mites on the Domesticated Honeybee. *Virginia Cooperative Extension* (*VCE*). 444–103. https://vtechworks.lib.vt.edu/handle/10919/50392.

Brandt, A., Grikscheit, K., Siede, R., Grosse, R., Meixner, M. D., & Büchler, R. (2017). Immunosuppression in Honeybee Queens by the Neonicotinoids Thiacloprid and Clothianidin. *Scientific Reports, 7*, 4673. https://doi.org/10.1038/s41598-017-04734-1.

Dainat, B., Evans, J. D., Chen, Y. P., Gauthier, L., Neumann, P. (2012). Dead or Alive: Deformed Wing Virus and *Varroa* destructor Reduce the Life Span of Winter Honeybees. *Applied and Environmental Microbiology*, *78*(4), 981–987. https://doi.org/10.1128/AEM.06537-11.

Flanagan, D., & Matsumoto, Y. (2016). The Ruby Programming Language (Japanese version translated by Takahiro Nagao under a supervisor of Shohei Urabe), issued the 8th printing of the first edition; O'Reilly Japan Publisher (Selling agency: Ohmsha, Tokyo), Tokyo, July 5, 2016. https://books.google.co.jp/books?hl=ja&lr=lang_ja|lang_en&id=jcUbTcr5XWwC&oi=fnd&pg=PR5&dq=Ruby+Programming+Language&ots=fLBht9bucG&sig=fJuPX7bnJv5MoBDzwAtH2JLpV-A#v=onepage&q=Ruby%20Programming%20Language&f=false.

Forfert, N., Troxler, A., Retschnig, G., Gauthier, L., Straub, L., Moritz, R. F. A., Neumann, P., Williams, G. R. (2017). Neonicotinoid pesticides can reduce honeybee colony genetic diversity. *PLoS ONE*, *12*(10), e0186109 https://doi.org/10.1371/journal.pone.0186109.

Fukuda, H., & Sekiguchi, K. (1966). Seasonal change of the honeybee worker longevity in Sapporo, north Japan, with notes on some factors affecting the life-span. *Japanese Journal of Ecology, 16*(5), 206–212. https://doi.org/10.18960/seitai.16.5_206.

Gregorc, A., Silva-Zacarin, E. C. M., Carvalho, S. M., Kramberger, D., Teixeira, E. W., Malaspina, O. (2016). Effects of Nosema ceranae and thiametoxam in *Apis mellifera*: A comparative study in Africanized and Carniolan honey bees. *Chemosphere*, *147*, 328–336. https://doi.org/10.1016/j.chemosphere.2015.12.030.

Khongphinitbunjong, K., Neumann, P., Chantawannakul, P., Williams, G. R. (2016). The ectoparasitic mite *Tropilaelaps mercedesae* reduces western honey bee, *Apis mellifera*, longevity and emergence weight, and promotes Deformed wing virus infections. *The Journal of Invertebrate Pathology*, *137*, 38–42. https://doi.org/10.1016/j.jip.2016.04.006.

Macedo P.A., Wu J., & Ellis, M.D. (2002). Using inert dusts to detect and assess varroa infestations in honey bee colonies. *Journal of Apicultural Research*, *41*(1–2), 3–7. https://doi.org/10.1080/00218839.2002.11101062.

Martin, S. J., Ball, B. V., & Carreck, N. L. (2013). The role of deformed wing virus in the initial collapse of varroa infested honey bee colonies in the UK. *Journal of Apicultural Research, 52*(5), 251–258. https://doi.org/10.3896/IBRA.1.52.5.12.

Nagata, I., & Yamada, T. (1973). On the parameter-seeking method of local composition equations. *Journal of Chemical Engineering of Japan, 6*(3), 215–219. https://doi.org/10.1252/jcej.6.215.

Omholt**,** S. W. (1988). Relationships between worker longevity and the intracolonial population dynamics of the honeybee. *The Journal of Theoretical Biology 130*(3,7), 275–284. https://www.sciencedirect.com/science/article/abs/pii/S0022519388800304.

Peng, Y.-C., & Yang, E.-C. (2016). Sublethal Dosage of Imidacloprid Reduces the Microglomerular Density of Honey Bee Mushroom Bodies. *Scientific Reports*, *6*, 19298. https://doi.org/10.1038/srep19298.

Reyes-Quintana, M., Espinosa-Montaño, L. G., Prieto-Merlos, D., Koleoglu, G., Petukhova, T., Correa-Benítez, A., & Guzman-Novo E**.** (2019). Impact of *Varroa destructor* and deformed wing virus on emergence, cellular immunity, wing integrity and survivorship of Africanized honey bees in Mexico. *The Journal of Invertebrate Pathology, 164*, 43–48. https://doi.org/10.1016/j.jip.2019.04.009.

Sammataro, D., Gerson, U., & Needham, G. (2000). Parasitic Mites of Honey Bees: Life History, Implications, and Impact. *Annu. Rev. Entomol.* **45**, 519–548. https://doi.org/10.1146/annurev.ento.45.1.519.

Schmid-Hempel, P., & Wolf, T. (1988). Foraging Effort and Life Span of Workers in a Social Insect. *Journal of Animal Ecology*, *57*(2), 509–521. https://doi.org/10.2307/4921.

Shi, J., Liao, C., Wang, Z., Zeng, Z., & Wu, X. (2019). Effects of sublethal acetamiprid doses on the lifespan and memory-related characteristics of honey bee (*Apis mellifera*) workers. *Apidologie*, *50*,

553–563. https://doi.org/10.1007/s13592-019-00669-w.

Solanki, C., Thapliyal, P., & Tomar, K. (2014). Role of Bisection Method. *International Journal of Computer Applications Technology and Research*, *3*(8), 533–535. https://ijcatr.com/archives/volume3/issue8/ijcatr03081009.pdf.

Strange J.P., & Sheppard W.S., (2001). Optimum Timing of Miticide Applications for Control of *Varroa destructor* (Acari:Varroidae) in *Apis mellifera* (Hymenoptera: Apidae) in Washington State, USA. *Journal of economic entomology*, *94*(6): 1324–1331. https://doi.org/10.1603/0022-0493-94.6.1324.

Straub, L., Williams, G. R., Vidondo, B., Khongphinitbunjong, K., Retschnig, G., Schneeberger, A., Chantawannakul, P., Dietemann, V., Neumann, P. (2019). Neonicotinoids and ectoparasitic mites synergistically impact honeybees. *Scientific Reports*, *9*, 8159. https://doi.org/10.1038/s41598-019-44207-1.

Tackenberg, M. C., Giannoni-Guzmán, M. A., Sanchez-Perez, E., Doll, C. A., Agosto-Rivera, J. L., Broadie, K., D. Moore, & McMahon, D. G. (2020). Neonicotinoids disrupt circadian rhythms and sleep in honey bees. Scientific Reports, 10, 17929. https://doi.org/10.1038/s41598-020-72041-3.

Tarek, H., Hamiduzzaman, M. M., Morfin, N., & Guzman-Novoa, E. (2018). Sub-lethal doses of neonicotinoid and carbamate insecticides reduce the lifespan and alter the expression of immune health and detoxification related genes of honey bees (*Apis mellifera*). *Genitics and Molecular* Research, *17*(2), gmr16039908. https://doi.org/10.4238/gmr16039908.

Vidau C., Diogon M., Aufauvre J., Fontbonne R., Viguès B., Brunet J.-L., Texier C., D. Biron, G., Blot N., Alaoui H. E., Belzunces L. P., & Delbac, F. (2011). Exposure to Sublethal Doses of Fipronil and Thiacloprid Highly Increases Mortality of Honeybees Previously Infected by Nosema ceranae. *PLoS ONE*, *6*(6), e21550. https://doi.org/10.1371/journal.pone.0021550.

Wang, H., Zhang, S.-W., Zeng, Z.-J., Yan, W.-Y. (2014). Nutrition affects longevity and gene expression in honey bee (*Apis mellifera*) workers. *Apidologie*, *45*, 618–625. https://doi.org/10.1007/s13592-014-0276-3.

Wolf, T. J., & Schmid-Hempel, P. (1989). Extra Loads and Foraging Life Span in Honeybee Workers**.** *Journal of Animal Ecology*, *58*(3), 943–954. https://doi.org/10.2307/5134.

Yamada, T., Yamada, K., & Wada, N. (2012). Influence of dinotefuran & clothianidin on a bee colony. *Japanese journal of clinical* ecology, *21*(1),10–23.

http://bijensterfte.nl/sites/default/files/Influence%20of%20dinotefuran%20&%20clothianidin%20on%20a%20bee%20colony_Jpn%20J%20clin%20Ecol%20Vol21,%20No1,%2010-23(2012).pdf

Yamada, T., Yamada, K., & Yamada, Y. (2018a). A clear difference in the impact on honeybee (*Apis mellifera*) colony between the two vehicles of sugar syrup and pollen paste. *Journal of Biological Series, 1*(5), 84–107. https://www.academiapublishing.org/journals/jbs/abstract/2018/Jul/Toshiro%20et%20al.htm.

Yamada, T., Yamada, Y., & Yamada, K., (2018b). Difference between the impact of the neonicotinoid dinotefuran and organophosphate fenitrothion on a bee colony in a long-term field experiment: an evidence. *Journal of Biological Series, 1*(3), 108–137. https://www.academiapublishing.org/journals/jbs/abstract/2018/Jul/Yamada%20et%20al.htm.

Yamada, T., Yamada, Y., & Yamada, K. (2018c). 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). *Journal of Biological Series, 1* (4), 187–207. https://www.academiapublishing.org/journals/jbs/abstract/2018/Oct/Yamada%20et%20al.htm.

Yamada, T., Yamada, K., and Apao, P. (2018d). Comparison of the long-term influence of a pesticide on a bee colony between neonicotinoids (dinotefuran, clothianidin) and organophosphate (fenitrothion) in Maui where there are neither harmful mites nor cold winter. *Journal Biological Series*, *1*(4), 156-186. https://www.academiapublishing.org/journals/jbs/abstract/2018/Oct/Yamada%20et%20al..htm.

Yamada, Y., Yamada, T., & Yamada, K. (2019). A mathematical model to estimate the seasonal change in apparent longevity of bee colony. *Scientific Reports, 9*, 4102. https://doi.org/10.1038/s41598-019-40725-0.

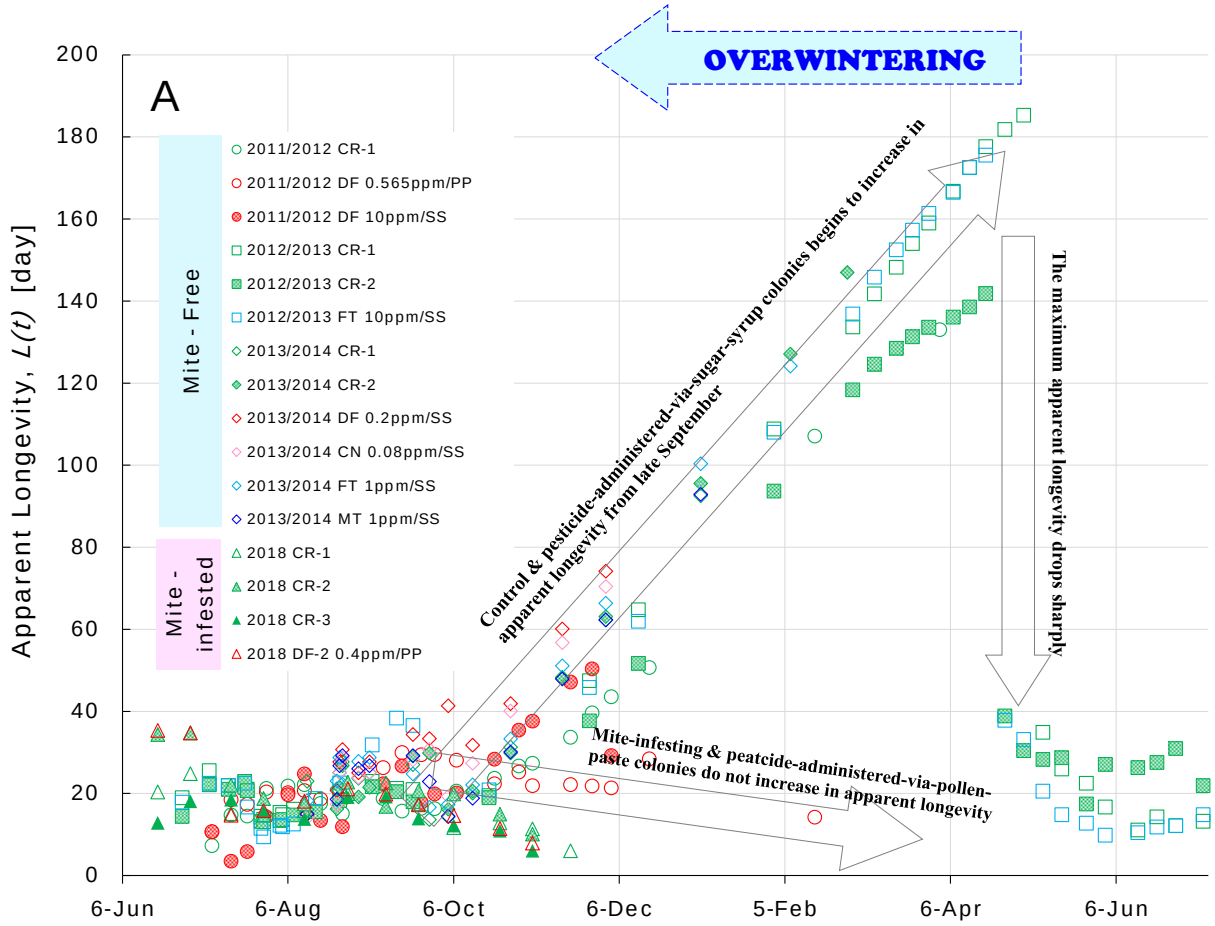
Yamada, T., & Yamada, K. (2020). Comparison of long-term changes in size and longevity of bee colonies in mid-west-Japan and Maui with and without exposure to pesticide, cold winters, and mites. *PeerJ*, *8*, e9505. https://doi.org/10.7717/peerj.9505.

Yamada, T. (2020). Seasonal Changes in the Size and Mite-Prevalence of A Bee Colony Exposed to Dinotefuran via Pollen Paste and Damaged by *Varroa* Mites. *Enliven: Journal of Dietetics Research and Nutrition*, *7*(1), 002. http://www.enlivenarchive-box.org/data/seasonal-changes-in-the-size-and-miteprevalence-of-a-bee-colony-exposed-to-dinotefuran-via-pollen-paste-and-damaged-by-varroa-mites-0.html.

Yang, E.-C., Chang, H.-C., Wu, W.-Y., & Chen, Y.-W. (2012). Impaired Olfactory Associative

Behavior of Honeybee Workers Due to Contamination of Imidacloprid in the Larval Stage. *PLoS ONE*, *7*(11), e49472. https://doi.org/10.1371/journal.pone.0049472

Yang, W., Tian, Y., Han, M., Miao, X. (2017). Longevity extension of worker honey bees (*Apis mellifera*) by royal jelly: optimal dose and active ingredient. *PeerJ*, *5*, e3118. https://doi.org/10.7717/peerj.3118.


A
OVERWINTERING
Apparent Longevity, L(t) [day]
200
180
160
140
120
100
80
60
40
20
0
6-Jun
6-Aug
6-Oct
6-Dec
5-Feb
6-Apr
6-Jun
Mite - Free
Mite - infested
2011/2012 CR-1
2011/2012 DF 0.565ppm/PP
2011/2012 DF 10ppm/SS
2012/2013 CR-1
2012/2013 CR-2
2012/2013 FT 10ppm/SS
2013/2014 CR-1
2013/2014 CR-2
2013/2014 DF 0.2ppm/SS
2013/2014 CN 0.08ppm/SS
2013/2014 FT 1ppm/SS
2013/2014 MT 1ppm/SS
2018 CR-1
2018 CR-2
2018 CR-3
2018 DF-2 0.4ppm/PP
Control & pesticide-administered-via-sugar-syrup colonies begins to increase in apparent longevity from late September
The maximum apparent longevity drops sharply
Mite-infesting & peatcide-administered-via-pollen-paste colonies do not increase in apparent longevity

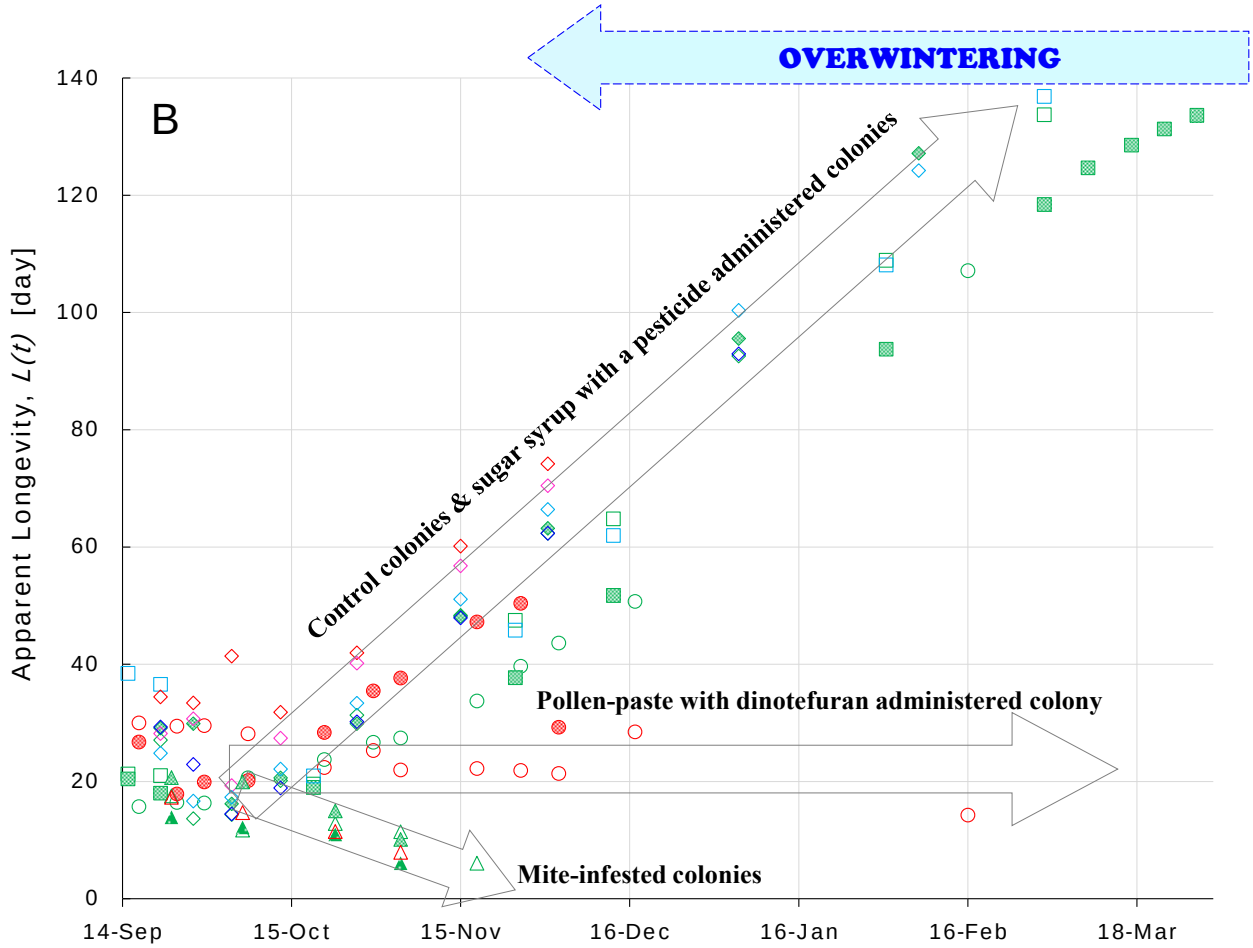

B
OVERWINTERING
Apparent Longevity, L(t) [day]
140
120
100
80
60
40
20
0
14-Sep
15-Oct
15-Nov
16-Dec
16-Jan
16-Feb
18-Mar
Control colonies & sugar syrup with a pesticide administered colonies
Pollen-paste with dinotefuran administered colony
Mite-infested colonies

Figure 1. seasonal changes in apparent longevities of honeybee colonies in four long-term field-experiments.

(**A**): General view. (**B**): Date-axis enlarged view. Apparent longevities were estimated by using the numbers of adult bees and capped brood obtained from four long-term field-experiments conducted in : 2011 to 2012 (T. Yamada et al., 2018a) for the colonies of "2011/2012 CR-1", "2011/2012 DF 0.565ppm/PP" and "2011/2012 DF 10ppm/SS"; 2012 to 2013 (T. Yamada et al., 2018b) for the colonies of "2012/2013 CR-1", "2012/2013 CR-2" and "2012/2013 FT 10ppm/SS"; 2013 to 2014 (T. Yamada et al., 2018c) for the colonies of "2013/2014 CR-1", "2013/2014 CR-2", "2013/2014 DF 0.2ppm/SS", "2013/2014 CN 0.08ppm/SS", "2013/2014 FT 1ppm/SS" and "2013/2014 MT 1ppm/SS"; 2018 (T. Yamada, 2020) for the colonies of "2018 CR-1", "2018 CR-2", "2018 CR-3" and "2018 DF-2 0.2ppm/PP". The colonies which had become extinct before wintering (before late November) are eliminated in this study. Where CR, DF, CN, FT and MT denote control (pesticide-free), dinotefuran, clothianidin, fenitrothion and malathion, respectively. “201x/201y” denotes the experimental period from 201x to 201y, “X Yppm” denotes the concentration “Y ppm” of the pesticide “X”. “syrup” and “pollen” denote SS and PP of vehicles through which the pesticide is administered into the honeybee-colony, respectively. See Supplementary Tables S3 to S6.

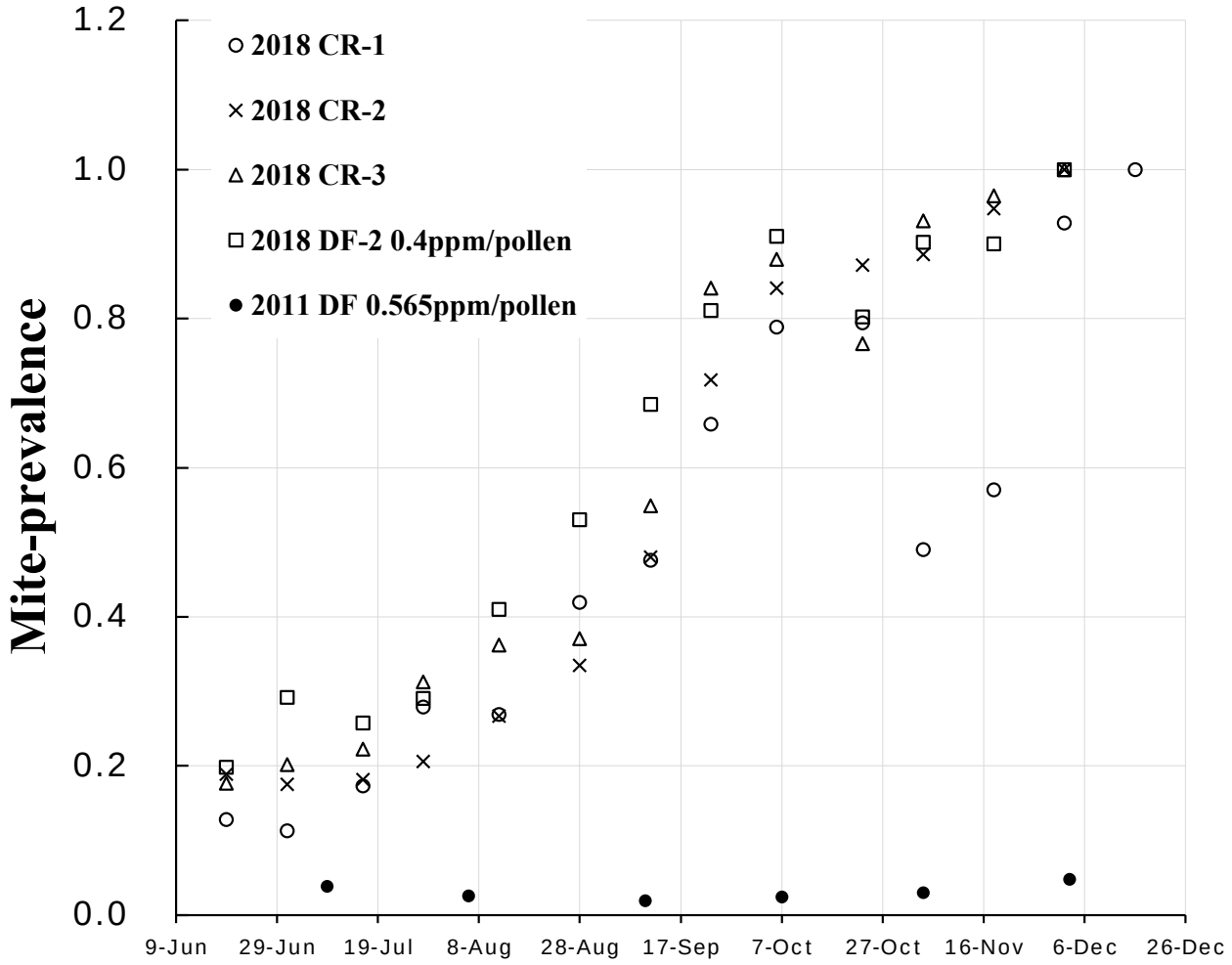


Figure 2. Comparison of mite-prevalence between the mite-infested colonies and the mite-free-&-toxic-PP colony.

2018 CR-1, 2018 CR-2 & 2018 CR-3: Pesticide-free control colonies in 2018-Experiment (CR-1, CrR-2, CR-3); 2018 DF-1, 2018 DF-2 & 2018 DF-3: Experimental colonies where dinotefuran is administered via PP in 2018-Experiment (DF-1, DF-2, DF-3). 2011/2012 DF: Experimental colonies where dinotefuran is administered via PP in 2011/2012-Experiment.

Mite-prevalence denotes the ratio of bees damaged by mites to total bees in each measurement interval. The apparent longevity of all colonies in this figure could not begin to extend around late September. Mite prevalence of all colonies (2018 CR-1, 2018 CR-2, 2018 CR-3, 2018 DF-1, 2018 DF-2, 2018 DF-3) in 2018-Experiment, where dinotefuran (neonicotinoid) were administered via PP, are about 50% in mid-September, about 80% in mid-October, and finally more than 90% (called mite-infested colony). On the other hand, the mite-prevalence of experimental colony in 2012-Experiment (2011/2012 DF) where dinotefuran was administered via PP is almost 2~3%, and at most less than 5%. See Supplementary Table S7.

Supplementary Material for

Neonicotinoids and *Varroa* mites force the extending of longevity in a bee colony during overwintering to forget

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This PDF file includes

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Supplementary Method S1; Supplementary Method S2; Supplementary Method S3

Supplementary Figures

Supplementary Figure S1; Supplementary Figure S2; Supplementary Figure S3; Supplementary Figure S4; Supplementary Figure S5; Supplementary Figure S6; Supplementary Figure S7; Supplementary Figure S8; Supplementary Figure S9; Supplementary Figure S10

Supplementary Tables

Supplementary Table S1; Supplementary Table S2; Supplementary Table S3; Supplementary Table S4; Supplementary Table S5; Supplementary Table S6; Supplementary Table S7

Supplementary References

References with an underscore for the first author are cited in the supplementary material, and the rest are cited in the text.

Supplementary Material for

Neonicotinoids and *Varroa* mites force the extending of longevity in a bee colony during overwintering to forget

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1 Materials and Methods

1.1 Supplementary Method S1: Determination method of apparent longevity

1.1.1 Mathematical model

Using the numbers of adult bees and capped brood obtained from long-term field experiments, the authors have proposed a mathematical model for estimating the apparent longevity of a honeybee colony at every observation-experiment interval. There are various day-age groups in the bee group, of which the theory seeks the day-age of the oldest living in honeybee group. The following is an overview of the mathematical model.

If we can estimate the apparent longevity of a honeybee colony, we can get more comprehensive information from field experiments. The authors have proposed a mathematical model that can estimate the apparent longevity from the numbers of adult bees and capped brood. Here a dynamic parameter called "apparent longevity", *L (t)*, defined in Equation (S1) is introduced, which depicts the characteristics of the "boundary" day-age where the average honeybee-worker disappears from the bee colony:

$$a(t) = \int_{T_{pupa}}^{T_{pupa}+L(t)} u(t - s)p(t - s)ds \qquad \text{(S1)},$$

where *a(t)* and *u(t)* are the number of adult bees and the number of newly capped brood per unit time *t*, respectively. *p (t)* is the eclosion rate from capped brood (pupae in comb-cells with a sealed entrance) at time *t*. T_{pupa} is the average period of capped brood, and the period of honeybees *Apis mellifera* is 12 days. If the bee colony is in a steady state, the worker bees die at the same age. *L(t)* represents the longevity of an adult bee. The age distribution of dying bees is given by the narrow Gaussian distribution.

Therefore, it is worth noting that Equation (S1) may provide an estimate of the longevity of a worker bee in a normal bee group.

In order to find the apparent longevity, *L(t)*, in Equation (S1), it is necessary to know *a(t)*, *u(t)* and *p(t)*. The values of *a(t)* and *u(t)* will be obtained from measurements in field experiments. Since it is difficult to directly measure *u(t)*, it is estimated from Equation (S2) using the *b(t)* value measured by the field experiment.

$$b(t) = \int_{0}^{T_{pupa}} u(t - s)[p(t - s)]^{s/T_{pupa}} ds \qquad \text{(S2)},$$

where *b (t)* is the number of capped brood at time *t*. Equation (S2) assumes that the pupa is removed as soon as it is dead in the comb cells.

By combining Equations (S1) and (S2), it is possible to characterize the honeybee group using a macroscopic amount on honeybees (the number of adult bees and number of capped brood) that are easy to get by experiments. Although *a(t)* in Equation (S1) and *b(t)* in Equation (S2) are continuous in the formula (it is a continuous function), it is actually impossible to measure them continuously in field experiments. Since the measurement date is discrete, Equations (S1) and (S2) were separated under the following assumptions.

1) The day-age distribution of capped brood on a measurement date is assumed to be discretely uniform during the capping period on the comb-cells (12 days), and to be constant between two consecutive measurement dates (in each measuring interval). These assumptions are equivalent to that the number of newly capped brood per day, $u(t)$, is constant between two consecutive measurement dates. If the number of capped brood measured on a measurement date is expressed in $b(t_k)$, the daily average number of adult bees that newly emerges from the capped brood between one measurement date and the next measurement date is given by $b(t_k)/T_{pupa}$.

2) The eclosion rate is assumed to be constant in two consecutive measurement dates.

3) It is assumed that the pesticide intake per larva in two consecutive measurement dates is assumed to be the same as the intake of pesticides per bee. In our experiments, it is not possible to directly measure the intake of pesticides per larva between two consecutive measurement dates. Therefore, the pesticide intake per of the larva is substituted for that the that per measurable adult bee.

In Equations (S1) and (S2), $a(t)$ and $b(t)$ are known values measured in field experiments, but $L(t)$, $u(t)$ and $p(t)$ are unknown. In two simultaneous equations, one of these three unknown variables must be known before these simultaneous equations can be solved. Fortunately, the eclosion rates, $p(t)$, at various imidacloprid doses has already been reported (Yang et al. 2012). Figure S1 shows the relationship between the imidacloprid dose and the eclosion rate (mean, minimum, maximum value) in a semi-logarithmic graph. The eclosion rate required for calculating the apparent longevity can be obtained using the mean eclosion rate curve in Figure S1.

Since the pesticide used in this experiment is dinotefuran, not imidacloprid, the dinotefuran concentration must be converted to the imidacloprid dose in order to use Figure S1. Therefore, the imidacloprid dose equivalent to the insecticidal ability of a certain dinotefuran concentration is estimated as follows.

Field experiment conducted by the authors (T. Yamada et al., 2012) shows that the insecticidal ability of clothianidin for honeybees is about 2.5 times that of dinotefuran (this is the same as the ratio of the recommended insecticidal concentration of dinotefuran for exterminating stink bugs to that of clothianidin).

It can be assumed that imidacloprid's ability to exterminate honeybees is about 2.5 times that of dinotefuran, since the recommended insecticidal concentration of imidacloprid for exterminating stink bugs is approximately the same as that of clothianidin. Pesticide intake per bee in two consecutive measurement dates can be estimated from the total pesticide intake and total number of adult bees between the measurement dates using the method proposed by the authors (T. Yamada et al., 2018a).

In the range of pesticide dosages in previous field experiments (see Table S1), as shown in Figure S2, the effect on the eclosion rate from bee pupae, $p(t)$, of the pesticide dose is small. In other words. there is little difference in calculated apparent longevity between when the eclosion rate changes with the pesticide dose per bee in the period of the interval

between arbitrary two consecutive measurement dates as expressed by $p(t) = f(pesticide\ dose)$ and when it is assumed that the eclosion rate is equal and constant to 0.9 of the control group.

Therefore, in this paper, the apparent longevity was calculated with a constant eclosion rate of 0.9 throughout the experiment as previously reported (T. Yamada & K. Yamada, 2020). Using the dataset at each measurement date obtained from the field experiment, the apparent longevity, $L(t)$, can be obtained by solving the discrete equation as shown in the previous report (Y. Yamada, 2019).

1.1.2 Determination procedure

The procedure for calculating the apparent longevity using the proposed mathematical model as described above will be described below. The unknown variables in the equations for determining the apparent longevity of a bee colony in each measurement (observational experiment) are the apparent longevity $L(t)$ and the number of the newly capped brood $u(t)$. Of these two variables, $u(t)$ can be obtained from Equation (S2). Of these two variables, $u(t)$ can be determined from Equation (S2).

Therefore, the $u(t)$ value determined from Equation (S2) is substituted to Equation (S1), and the unknown variable $L(t)$ in Equation (S1) is determined by an iteration method such as the Bisection method (Solanki et al., 2014), the Simplex method (Nagata & Yamada, 1973) and so on as below. While changing the value $L(t)$, a certain value $L(t)$ is repeatedly substituted for Equation (S1). And then, the calculations of discretization equations for Equations (S1) and (S2) are repeated until a difference between the calculated value of the number of adult bees $a(t)$ obtained by the iteration method and the measured value of the number of adult bees by a field experiment reaches a given convergence criterion value (0.0001 in this paper) or less.

In the above calculation to seek the apparent longevity $L(t)$, actual measurement data can be obtained only for each measurement date. Therefore, the apparent longevity $L(t)$ can be sought after the discretization of the equations while taking account of the life cycle from oviposition to eclosion of the honeybee (egg period 3 days; larval period 6 days; pupa (capped brood) period 12 days).

In this study, the apparent longevity $L(t)$ is determined by the Bisection method (the convergence radius of $L(t)$ is 0.0001 in this work). The calculations were performed using the Ruby programming language (Flanagan & Matsumoto, 2016). The flowchart is shown in Figures S3 and S4. Figure S3 is a whole flow diagram for determining an apparent longevity $L(t)$, and Figure S4 shows a procedure for seeking the apparent longevity $L(t)$ in Equation (S1) by the Bisection method. These flowcharts are described in a little more detail below.

Here, we will explain the whole flow of the calculation to seek the apparent longevity (Figure S3). The eclosion rate p_k of a honeybee colony is calculated using the relationship between the dose of imidacloprid per larva and the eclosion rate (Figure S1) (Yang et al. 2012). Our previous experiments were conducted using pesticides other than imidacloprid. In addition, the intake of a pesticide by an adult bee can be directly calculated from experimental

data, but that by alarva cannot. Larvae cannot directly take food (sugar solution, pollen paste) and adult bees (nurse bees) usually feed larvae on food. Therefore, it can be supposed that the intake of a pesticide of one larva is almost the same as that of an adult bee. In addition, it is assumed that the pesticide intake per bee is the same regardless of the role (nursing, forming) in adult bees. In other words, "dose of imidacloprid per larva" is equivalent to "intake of imidacloprid per bee".

By the way, imidacloprid and other pesticides (dinotefuran, clothianidin, fenitrothion, malathion) have different insecticidal ability for bees, respectively. With considering the insecticidal ability of each pesticide, each pesticide intake is converted into an intake of imidacloprid intake, and the ratio of the dosage concentration of each pesticide to the bee colony is adjusted so that the insecticidal activity to exterminate stink bugs, which are common pests in Japan, is the same (T. Yamada & K. Yamada, 2020; T. Yamada et al, 2018b; 2018c).

In a honey bee colony during the period of each interval between two consecutive measurement dates, the total amount of a pesticide ingested by adult bees and the total number of adult bees involved in the pesticide intake are different at each interval. Incidentally, the total number of adult bees during the period of each interval can be obtained by the sum of the both numbers of adult bees that will newly eclose from capped brood and the number of adult bees existing from the beginning of each interval. Exceptionally, the total number of adult bees during the period of the final interval is obtained by the sum of the both numbers and the number of capped brood at the end of experiment. Because the capped brood can be presumed to have already ingested the pesticide.

Therefore, it is necessary to determine the intake of a pesticide per bee for each interval (see below for details). If the intake of a pesticide per bee at each interval is known, the eclosion rate p_k in each interval can be estimated from Figure S1.

In a honeybee colony, by dividing the total amount of pesticide intake (TI_k) ingested by the honeybee colony between the two consecutive measurement dates by the sum (N_k) of the total number of adult bees (B_k) eclosing between the $(k-1)^{th}$ measurement date to the kth one and the measured number of adult bees (a_{k-1}) already existing on the $(k-1)^{th}$ measurement date, the average intake of a pesticide per bee ($I_k = TI_k / N_k$) can be determined. In each measurement interval, the intake of a pesticide per bee is converted to that intake of imidacloprid per larva under the assumptions mentioned above, and then the eclosion rate p_k is estimated from Figure S1. In the pesticide-intake range in our field experiments, the apparent longevity $L(t)$ calculated with the estimated eclosion rate p_k depending on the pesticide intake differs little from the apparent longevity calculated with the constant eclosion rate of 0.9 (Figure S2) on the assumption that the eclosion rate of pesticide-intake colony is independent of pesticide intake and equal to the eclosion rate for the control colony. Therefore, the eclosion rate of 0.9 is used to calculate the apparent longevity in this paper.

Next, taking into account the life cycle of the bee (egg period of 3 days; larval period of 6 days; pupa (capped brood) period of 12 days), the simultaneous equations that discretize

Equation (S2) on the kth measurement date is solved, and the number of newly capped brood u_k is determined (**STEP 1**).

The number of newly capped brood u_k determined as described above is substituted for Equation (S1). Then, while changing the apparent longevity L_k on the kth measurement date with the Bisection method, calculations are repeated till both a difference between the measured number of adult bees and the calculated one and a difference between the old value of L_k and the new one becomes both less than 0.0001. When these convergence criteria are satisfied, L_k is the determined value. After iterating the above calculations and completing them for all the number of experiments (k= 1, 2, 3, ⋯ , N) (**STEP 2**), the calculated results are printed.

When determining the apparent longevity L_k by the Bisection method, first L_k is increased with a rough step size (0.5 in this study), and two points (L_k values) where the signs of the Y ($(a_k)_{\text{measured}} - (a_k)_{\text{calculated}}$) are reversed are found (Figure S4). The two L_k values before and after the sign is reversed are sandwiched, and the step size is further reduced, and the calculations are repeated until both the Y is closest to zero with the Bisection method and the absolute value of ($L_{k,\ new}$ - $L_{k,\ old}$) becomes less than 0.0001. When the above criteria are satisfied, the value of L_k is regarded as the determined apparent longevity.

1.2 Supplementary Method S2: Field experiments

1.2.1 Precautions for obtaining accurate measurements

Both of the numbers of adult bees and capped brood, which are very important for obtaining apparent longevity by use of a mathematical model and can be measured in the experiment, are deeply involved through the life cycle of honeybees each other. If the measurement accuracy of either number is poor, a contradiction will arise between these two measurements. For example, when some of adult bees have gone out foraging, the number of adult bees will underestimate the actual number existing in the honey bee colony. In this case, because there is an unreasonable difference in numbers between the two, apparent longevity cannot be accurately obtained when solving simultaneous equations in a mathematical model, or cannot be sometimes even determined because the simultaneous equations cannot be solved. Therefore, experiments are conducted while paying attention to the following points to measure accurately the numbers: The experiment is started immediately after dawn to minimize the number of adult bees out of the hive-box. The comb-frame with bees is pulled out from the hive-box as gently as possible, when taking picture, to prevent the bees from flying away, and is gently set on the photo stand. The experiment is conducted while avoiding a windy or rainy day, judging from the weather forecast, because of preventing the bees from becoming restless. In more details, see the previous paper (T. Yamada, 2020).

1.2.2 Materials used in the field experiments and their preparation

The following materials were used in the long-term field experiments.

【**Pesticides**】

1) Dinotefuran (neonicotinoid pesticide) (DF): Starkle Mate® (10% DF; Mitsui Chemicals Aglo, Inc., Tokyo, Japan).
2) Clothianidin (neonicotinoid pesticide) (CN): DANTOTSU® (16% clothianidin; Sumitomo Co. Ltd., Tokyo, Japan).
3) Fenitrothion (organophosphate pesticide) (FT): SUMITHION® emulsion (50% fenitrothion; Sumitomo Co. Ltd., Japan) was used.
4) Malathion (organophosphate pesticide) (MT): MALATHON® emulsion (50% malathion; Sumitomo Co. Ltd., Japan).

【**Foods** which are sometimes used as vehicles to administer a pesticide to a bee colony】

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

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

3) SS with pesticide of a desired concentration: It was produced using 60% SS solution containing the desired concentration of pesticide and sugar. The prepared SS containing pesticide in a 10 L-container was blocked from light using a black bag and stored in a refrigerator.

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

1.2.3 Preparation for field experiment

Field experiments were conducted five times at the resort in mid-west Japan (Shika-machi, Hakui-gun, Ishikawa prefecture, Japan). Four field experiments (T. Yamada et al., 2012; 2018a; 2018b; 2018c) were conducted at the same location, while the remaining one field experiment (T. Yamada, 2020) was conducted at another location nearby. In addition, another field experiment was conducted from 2014 to 2015 in Maui, U.S.A. (T. Yamada & K. Yamada, 2020), and that is, six field experiments were conducted so far. The experimental environment, experimental methods, and publication outlines of four field experiments other than the field experiment conducted in Maui (T. Yamada & K. Yamada, 2020) where there is no cold season and the field experiment conducted in 2010 (T. Yamada et al., 2012) when the high accurate measurement method was not established yet, are summarized (Table S2).

In field experiments to investigate the effects of pesticides on bee colonies, it is necessary to minimize the effects of pesticides other than the pesticides administered in the experiments. Therefore, we tried to make the area around the experimental site as pesticide-free as possible. In other words, in the long-term field experiments in the Midwest of Japan, a water-feeding spot and foraging ground for bees were built in the immediate vicinity of the experimental site. Specifically, a system was installed that automatically supplies fresh water from a large tank. And, in a pesticide-free environment, new plants (rape blossoms *Brassica napus*, hairy vetch *Vicia viliosa Roth.*, white clover *Trifolium repens*, Chinese milk vetch *Astragalus sinicus*, wingstem *Verbesina alternifolia* and so on）and fruit-bearing trees （chestnut *Castanea crenata*, persimmon *Diospyros kaki*, mulberry *Morus alba*, Cherry tree *Prunus avium,* Japanese plum *Prunus salicina*, pawpaw *Asimina trilob*, etc.）were planted and cultivated (T. Yamada et al., 2018a). In addition, a long-term field experiment in the Maui, U.S.A. was conducted in woods of macadamia nuts grown without pesticides (T. Yamada & K. Yamada, 2020).

Here, we will introduce the experimental preparation concretely. Each hive-box was arranged at intervals of 80 cm, with its entrance facing south, on a stand of about 20 cm in height, and then a large PVC tray with many perforations of 3mm in diameter to prevent water from collecting was placed between the hive-box and the stand for the measurement of the number of dead bees.

While keeping in mind that the observation results of the photo images are analyzed, various measures were taken to prevent errors in field experiments. For example, in order to be able to infer the experimental contents of the bee colony in the hive-box, a large display tag was attached at the top of the front of each hive-box. A label was attached to the bottom of the hive-box or on four walls, displaying which part of the hive-box it was. Moreover, several labels displayed so that the position in the hive-box of each comb-frame and the front and back of the comb-frame could be seen were attached to the pier of the comb-frame. In addition, the name of the hive-boxes to be administered was pre-written on the sugar syrup (SS) container and the pollen paste (PP) tray so that the hive-boxes to be administered could be found.

1.2.4 Procedure for field experiment

The most important data in the field experiment of honeybee colony are both the number of adult bees and capped brood, which are indicators of the size, strength, vitality and power of the colony. To obtain these data as accurately as possible, the field experiment was carried out by the following procedure.

STEP-1) To avoid being stung by a bee, dress firmly with protective measures, and conduct experiments.

STEP-2) Prepare experimental tools, recording paper, containers with SS, trays with PP, camera, the empty hive-box or the like.

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

STEP-4) Count the number of dead bees in a large tray placed under the hive-box while picking up with tweezers (the number of dead bees outside the hive-box). Throw away what you've finished counting out of the tray. It should be noted that the number of dead bees in the hive-box is counted during the internal inspection of the hive-box. The number of dead bees inside and outside the hive-box is summed up and recorded as the number of dead bees on the date of the measurement.

STEP-5) Take a picture of the front of the hive-box on whose front the label for displaying the content of the hive-box is attached before the inspection of the inside of hive-box in order to make sure that the experiment target of the photography is not wrong.

STEP-6) Open the hive-box and remove the cloth that covered the top of the comb-frame of the hive-box, and take a whole photograph of the top of the hive-box. In addition, gently return the bees attached to the cloth in the hive-box not to prevent them from escaping from the hive-box.

STEP-7) The tray with PP and the container with SS (tray, feeding frame), which were put in the hive-box on the previous experimental date, are taken out from the hive-box, measuring the remaining amounts of PP and SS by the upper dish balance. Incidentally, when taking out the containers and the tray from the hive-box, gently return the bees attached to the container in the hive-box.

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

STEP-9) After taking pictures of both sides of all comb-frames with bees, take pictures of the four sides and the bottom in the hive-box in order to determine the number of adult bees left in the hive-box. If adult bees are outside the hive-box as well as inside the hive-box (sometimes near the entrance of the hive-box at tropical nights), take a picture to count the

number. The sum of these numbers of adult bees are regarded as the number of adult bees of the relevant colony (hive-box).

If you find a queen bee when you're taking a picture of a comb-frame, take its picture, if you cannot find it, check for the presence or absence of the queen on the comb-frames and inside the hive-box a few times. If the presence of the queen bee cannot be confirmed even after a few-times confirmations, after checking the photographs taken on that experimental date with expanding them, check again the queen bee in that hive-box on the next experiment. If you cannot find a queen bee at the next check, determine the absence or absence of a queen bee in the relevant hive-box after judging from the situations on queen cells, oviposition, the larvae, etc.

STEP-10 Once the number of adult bees has been measured and the queen has been confirmed, the bees attached to the comb-frame are shaken off from it to make a comb-frame without bees in order to determine the number of capped brood in each bee colony. In this way, the photographs on both sides of the comb frame without bees make it very easy to count the number of capped brood. The specific steps are described below.

STEP-11) In Take out the comb-frame with bees that had been placed in the spare hive-box in original numerical order, while gently shaking the bees off the comb-frame in the hive-box, and return all the bees attached to the comb-frame to the original hive-box. After taking pictures of both sides of the comb-frame without bees, return the comb-frame without bees to the original hive-box. Repeat the above process for all comb-frames.

By the way, at the point when you return a few bee-free comb-frames from the spare hive-box to the original hive-box, move the queen-cage containing the queen bee from the top side of the comb- frame of the spare hive-box to the top side of the comb-frame of the original hive-box. Once you have taken photos of both sides of all the comb-frame without bees, and have returned all the comb-frames from the spare hive-box to the original hive-box, remove the queen bee from the queen cage and return it to the original hive-box.

STEP-12) When taking a picture of the comb-frame without bee, you may discover some abnormality, such as queen-cells, *Varroa* mites, wax-moth larvae and so on. In this case, keep taking pictures of them. These anomalies are not limited to this situation, and if you find anything, record it in photos as evidence.

STEP-13) If there is a suspicion of the absence of the queen bee (death or disappearance, etc.), the division or escape of the bee colony, the attack of the giant hornet, the spread of mites, the infection of chalk disease, foulbrood, etc., the impact on the experiment will be significant, so if you find such an event during experimental observation, record it as a photographic image and record the detailed observation results in the laboratory notebook.

STEP-14) After covering the cloth on the top of comb-frames, put new SS and PP in the corresponding hive-box, the measurement is ended after the hive-box is covered by its lid.

STEP-15) After completion of the experiment, the test contents are confirmed and described in the laboratory notebook.

1.3 Supplementary Method S3: Counting methods of adult bees, capped brood, mite-damaged bees

Using the photographs taken as described above, it was possible to determine the numbers of adult bees, capped brood and mite-damaged bees. Counting such numbers from photographs is an extremely difficult task. Therefore, with the cooperation of Mr. Yoshiki Nagai of Nanosystem Co., Ltd., Kyoto, Japan (http://nanosystem.jp/firm.htm), which develops image processing software, the author has developed computer software in 2012 that automatically counts the relevant numbers from photographic images, and improvements have been made to the software since. The accuracy and operation of the counting have been greatly improved. By using this software, the time and accuracy for counting such numbers have been significantly improved, compared to the amount of work required to count by hand directly from the original photo.

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

1.3.1. Software developed to assist in accurately counting the numbers of adult bees, capped brood, and mite-damaged bees

This software was developed to count the numbers of adult bees and capped brood accurately. The following explains how to count the number of adult bees present in the image of a comb-frame and how to count the number of capped brood using the image read.

The numbers of adult bees and capped brood are counted with software using the image from a photo imported into the computer. The number of adult bees in the colony is obtained by summing the numbers of adult bees on both sides of all the comb-frames with the bees and the number of remaining adult bees in the hive-box without the comb-frame. The number of capped brood in a colony can be obtained by summing the numbers of capped brood on both sides of all the comb-frames without bees. The number of adult bees damaged by the *Varroa* mite can be obtained from the same photo used to count the adult bees. How to count bees damaged by *Varroa* mites is described in a separate section. The measurement results obtained by this method, as long as the image remains, can be verified by anyone at any time. In the following sections, the determination procedure of the numbers of adult bees and capped brood, and that of the number of mite-damaged bees, is described separately.

1.3.2 Determination procedure of the numbers of adult bees and capped brood

STEP-1) The photo image is automatically binarized by the software. Next, the area to be measured is set on the image by using the difference in brightness on the image between the place where adult bees exist and that where they do not, the difference in brightness between the place where capped brood exist and that they do not, and the difference in the features of the adult bee and the capped brood. The number of the identified adult bee or the number of the identified capped brood is counted in the area to be measured of the photo image.

STEP-2) In order to count as accurately as possible, before the binarization process of the image, divide the entire image into several areas that seem to have the same threshold value in each area (up to 4 areas) as below. Then, enter the optimal threshold for each divided area (the maximum number of divided areas 4, the split shape is optional).

Examples of division: (1) Distinguish between the capped honey area and the capped brood area. (2) Distinguish between areas where there are few bees and areas where bees are dense. (3) Distinguish between different areas of contrast or lightness (e.g., the part where the light hits and the part of the shadow).

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

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

STEP-5) After automatic counting by the computer, using the same software, switch to manual count operation, and then correct the count error at the time of automatic counting.

Therefore, using a large screen monitor, while further enlarging the image, mark-mistakes of adult bees and capped brood during automatic counting (duplicate count, counting things that are not the object) and forgetting to mark (due to the overlap of bees, the blurred image, the extreme differences in light and dark, etc.) are corrected while visually checking. All possible efforts on this correction are exerted for obtaining the number of adult bees or the number of capped brood as accurate as possible (most important and serious work).

It should be noted that, even when the software is manual counting, by the removal and grant of the mark, automatically change the number of bees or capped brood. You can resume this fix at any time. If it is determined that the correction is complete, it moves to the measurement of the next new image.

STEP-6) When posting the measured number of adult bees or capped brood in a separate table, call the measured image, check again in the enlarged image, after checking whether there is a count error (correct if you find a mistake), the number is posted to the separate table.

The sum total of the numbers of adult bees on all combs and those of adult bees on four walls and bottom in the hive-box becomes the number of adult bees in the bee colony at a certain measurement date. In a similar manner, the sum total of the numbers of capped brood of all combs, becomes the number of capped brood in the bee colony.

1.3.3 Counting procedure of the number of mite-damaged bees

Various sampling methods for *Varroa* mites in a honeybee colony, such as the sticky board method, the roll method, and the powder sugar shake method, have been reported (Strange & Sheppard, 2001; Macedo et al., 2002; Barlow & Fell, 2006). With these methods, it is impossible to determine the total number of *Varroa* mites in a bee colony.

It is presumed that the impacts of *Varroa* mites on a bee colony appear directly on the adult bees with damage caused by the mites. This section aims to measure the total number of bees damaged by mites, not the total number of mites in the bee colony.

To count the number of mite-damaged bees as accurately as possible, the images and software used to count the number of adult bees were used again. Using this counting technique for the number of adult bees, it is relatively easy to count *Varroa* mites on an adult bee. On the other hand, it is very difficult to count *Varroa* mites that causes damage to a brood in a comb cell. If any damage is received from *Varroa* mites during the brood stage, there should be a trace of the damage even if the brood becomes an adult bee. An adult bee with traces of its brood stage is considered to be a mite-damaged bee. For example, it is conceivable that a mite may fall out during the development of an adult bee. So, we checked to see if there were any traces of mites left on the adult bees. Therefore, assuming that such traces confirm the presence of mites, we checked the adult bees for traces of mites.

The criteria for the determination of such traces were created based on the information obtained by enlarging the image as is described below. The examples certified to be mite-damaged bees are shown in Figure S5 (T. Yamada, 2020). According to this criterion, using the photographic image taken for the adult number measurement, it is possible to evaluate the situation of the *Varroa*-mite damage among almost the total number of adult bees in the bee colony.

Therefore, the total number of both adult bees with *Varroa* mites and those with traces of *Varroa*-mite damage was determined manually with the software used to measure the number of adult bees while enlarging the adult bee number measurement image. The measurement of the number of mite-damaged bees was a very difficult task and took considerable time (about 6 months). To eliminate the errors between operators, this measurement was carried out by only one person. Figure S6 shows the seasonal changes of mite-damaged bees in a bee colony measured according to the following criteria.

1.3.4 Criteria for determining whether or not it is a mite-damaged bee

Just before capping a comb cell where a larva lives, female *Varroa* mites enter into the comb cell, and then lay eggs and multiply in it. Therefore, we determined that it would be impossible

to count the number of mites infesting bee-colony, directly.

In this study, rather than a method of directly counting the number of *Varroa* mites in the bee colony, it is assumed that the number of bees damaged by mites is equivalent to the degree of the bee colony damage caused by mites. The number of mite-damaged bees on the enlarged photographic image of the comb with bees taken to measure the number of adult bees, using the software developed to count the numbers of adult bees and capped brood mentioned above, is manually counted while referring to Figure S5 according to the following criteria.

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

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

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

By the way, there are cases when adult bees have not only traces of mites, but also mites themselves. Of course, in that case, count the number of mites attached to the adult bees.

The specific criteria for judgment are shown below. It is suspected that the three-dimensional one attached to a bee, which is small and round (including the ellipse), is a mite in the color of the red-brown system (including the oval) and red-brown system (including the semi-transparent). A suspected mite is determined whether it is a mite based on the criteria below described and Figure S5, while being examined from various angles, using an enlarged image. If a suspected mite cannot be determined by all means, it is considered a mite or a trace of the mite.

1) What appears to be planar rather than three-dimensional in the vicinity of the neck is considered to be an innate pattern of a bee appearing when the bee bends its neck or a shadow of other bee wings, etc., and is not considered damage by mites.

2) Tiny stuff with colors attached to adult bees other than red-brown and black (especially whitish ones) are regarded as pollen.

3) What the wings appear three-dimensional like wrinkles deformed is regarded as a shadow due to the shrinkage of the wings. However, the cause of the shrinkage of the wings is a large possibility of deformed wing virus mediated by *Varroa* mites. The state of the shrinkage of the wing is scrutinized on the image to determine whether the shrinkage is due to mites.
4) If a bee with shrunk or missing wings has traces of a mite bite, it is considered a mite-damaged bee, even if it is not possible to confirm the presence of a mite.
5) If you can't distinguish between mites and pollen or garbage, you should consider them mites. However, if it is clear that it is other than mites such as pollen and garbage, exclude it.
6) Planar ones are usually determined to be not mites. However, after scrutiny, exceptionally, it may be considered a mite-damaged bee.
7) Except for those that seem to be the rise of muscles, etc.
8) The difference between a mite and garbage is judged by shape, color, surrounding situation, etc., and it counts unnatural things like mites (Subjective, but count even what looks like a mite).
9) Traces bitten by mites (swelling by the bites) are also thought to be damage to bees.
10) What appears to be a mite, regardless of the number of traces bitten by mites, is treated as one mite-damaged bee.
11) Traces of a mite fallen out from a bee are treated as a mite-damaged bee.
Just before the larva's cell is capped, the mites lay eggs in the food in the cell, since the mites hatched from the eggs grow while sucking the body fluid of the bee's larva in the capped cell, it is almost impossible to find almost a mite even in the open cell. Therefore, it was decided not to evaluate the mites in the cells.
Mites are present in various places in the hive-box, shape is also easy to mistake for garbage and pollen, etc., also present in the invisible place (especially in the comb-cell), although the absolute number of mites is not known, this method to evaluate by the number of adult bees damaged by mites is believed to be a qualitative indicator of the number of presence of mites. Although the number of mites in the cell cannot be known, the infestation state of mites in the bee colony will be reflected in the number of mites adhering to the adult bee.
This method includes an uncertain element, under the above criteria, by measuring in the same person, and relative comparison of the number of mites between each bee colony, to some extent the time course of each bee group it is considered to represent. By the way, whenever a doubt occurs, again, using the image for the adult bee number measurement taken, recount.

2 Supplementary Figures (Figures S1 to S10)

Supplementary Figure S1. Change in eclosion rate of honeybees with imidacloprid dosage.

Supplementary Figure S2. Comparison of apparent longevity between a variable eclosion rate, $p(t)= f$ (dinotefuran- intake per bee), and a constant one, $p(t)= 0.9$.

Supplementary Figure S3. Flowchart to find the apparent longevity, $L(t)$: Mainstream scheme.

Supplementary Figure S4. Flowchart to find the apparent longevity, $L(t)$: Bisection method.

Supplementary Figure S5. Images of mite-damaged bees.

Supplementary Figure S6. Number of mite-damaged bees and mite-prevalence.

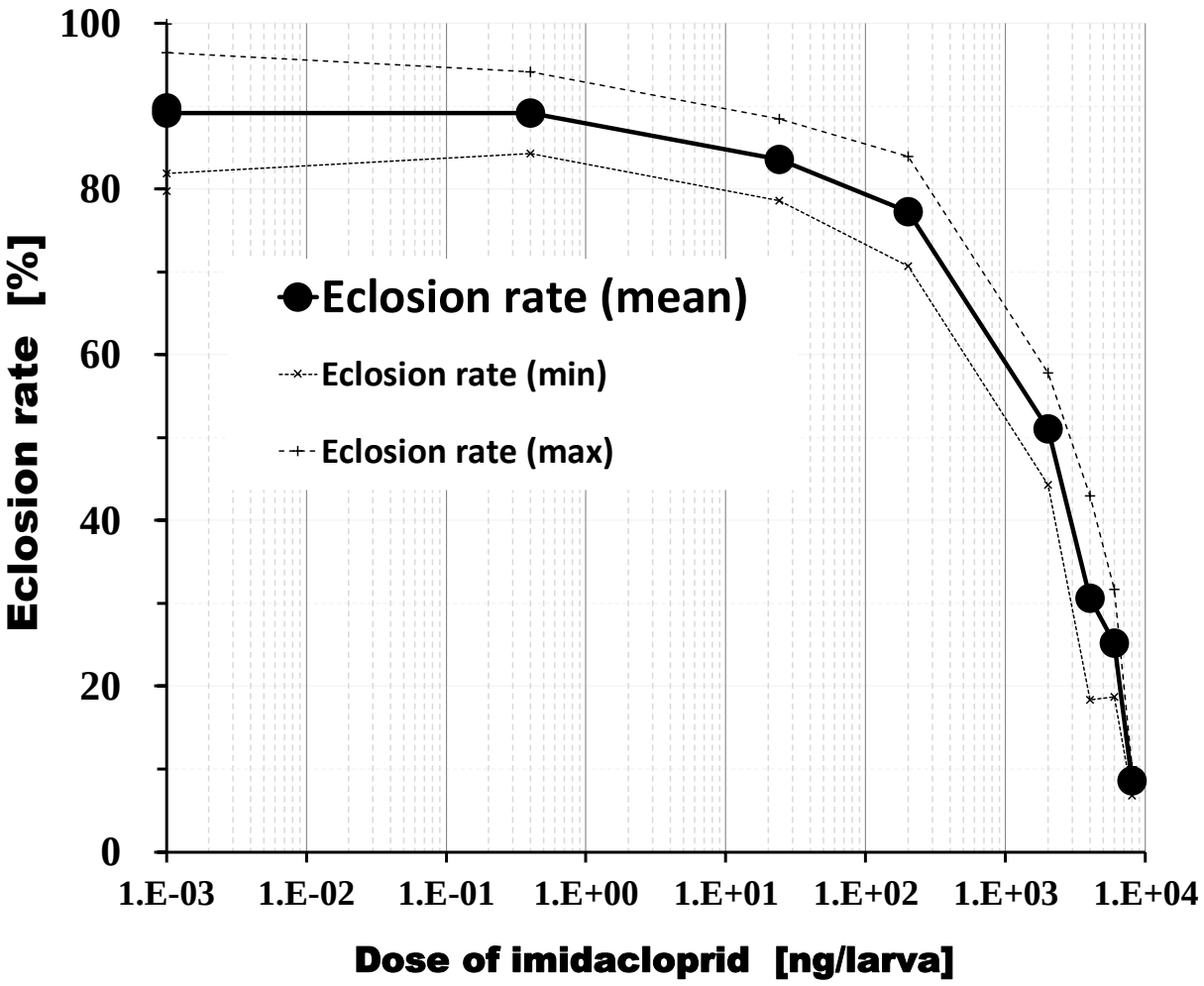
Supplementary Figure S7. Apparent longevity of 2011/2012-Experiment.

Supplementary Figure S8. Apparent longevity of 2012/2013-Experiment.

Supplementary Figure S9. Apparent longevity of 2013/2014-Experiment.

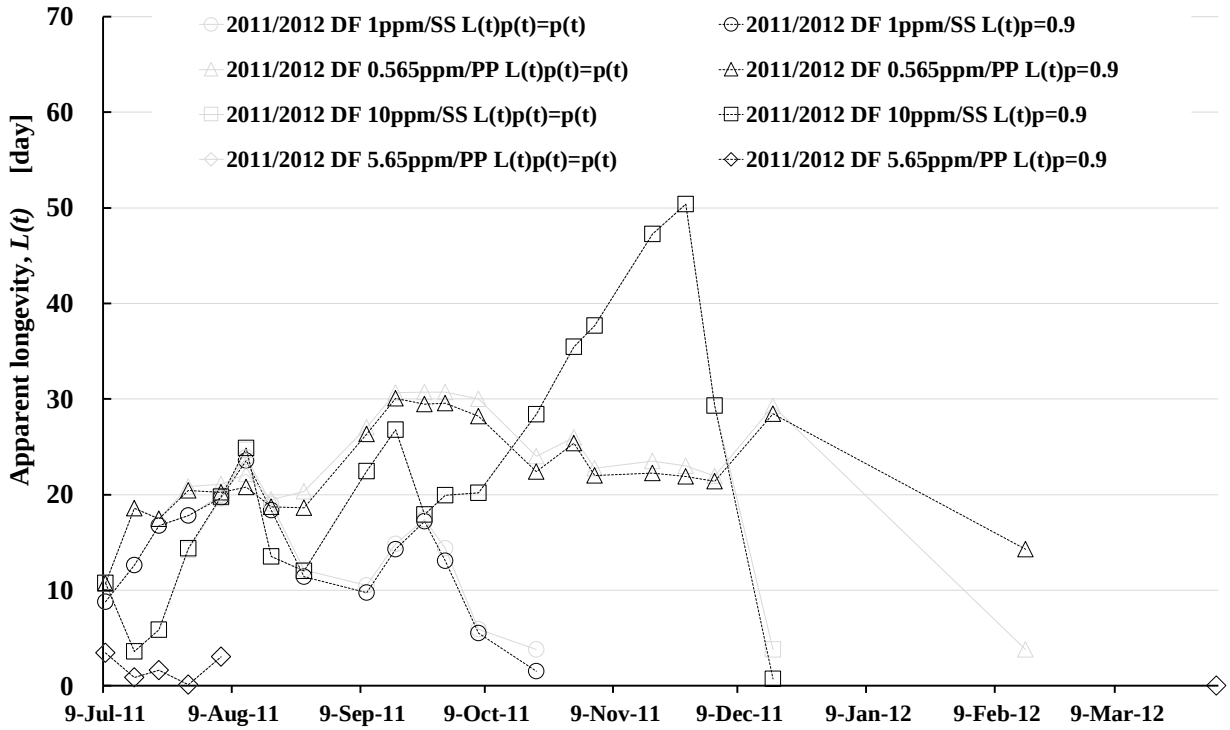
Supplementary Figure S10. Apparent longevity of 2018-Experiment.

Supplementary Figure S1. Change in eclosion rate of honeybees with imidacloprid dosage.
The source data on the relation between dose of imidacloprid and eclosion rate of honeybee are as follows (Yang et al. 2012): Eclosion rates are; 89.82±10.10 and 89.17±7.26 % for the control group; the rates are 89.20±4.90 % at the imidacloprid concentration of 0.4 ng/larva, 3.54±4.92 at that of 24 ng/larva, 77.29±6.64% at that of 200 ng/larva, 51.04±6.78 at that of 2000 ng/larva, 30.63±12.31 % at that 4000 ng/larva, 25.21±6.47 % at that of 6000 ng/larva and 8.54±1.72 % at that of 8000 ng/larva, respectively. Since Semi-log graphs cannot handle zero values, the pesticide concentration of the control group was set to 0.001 ng / larva for convenience.


100
80
60
40
20
0
Eclosion rate [%]
Eclosion rate (mean)
Eclosion rate (min)
Eclosion rate (max)
1.E-03
1.E-02
1.E-01
1.E+00
1.E+01
1.E+02
1.E+03
1.E+04
Dose of imidacloprid [ng/larva]

Supplementary Figure S2. Comparison of apparent longevity between a variable eclosion rate, *p(t)=f*(dinotefuran-intake per bee), and a constant one, *p(t)*=0.9.

To estimate the apparent longevity of honeybee colony were used the numbers of adult bees and capped brood measured in the field experiment conducted in 2011 to 2012 (T. Yamada et al., 2018a). $L(t)_{p(t)=p(t)}$ shows the apparent longevity was calculated with the variable *p(t)* changing with the pesticide intake per bee in a period between two consecutive measurement dates; where the ranges of *p(t)* are 0.79–0.9 for the "2011/2012 DF 1ppm/SS $L(t)_{p(t)=p(t)}$" colony; 0.84–0.9 for the "2011/2012 DF 0.565ppm/PP $L(t)_{p(t)=p(t)}$" colony, 0.8–0.9 for the "2011/2012 DF 10ppm/SS $L(t)_{p(t)=p(t)}$" colony, 0.835–0.9 for the "2011/2012 DF 5.65ppm/PP $L(t)_{p(t)=p(t)}$" colony, respectively. Incidentally, the intake of dinotefuran (DF) by one honeybee in a period between the observation interval was obtained according to the procedures reported in the previous paper (T. Yamada et al., 2018a). $L(t)_{p(t)=0.9}$ shows the apparent longevity was calculated with the constant *p(t)* of 0.9.

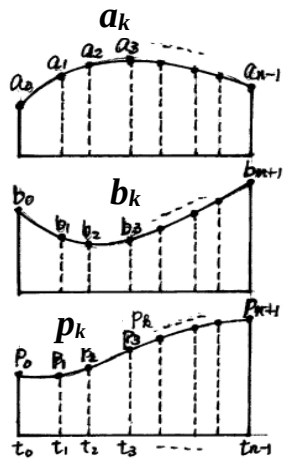


Supplementary Figure S3. Flowchart to find the apparent longevity, *L(t)*: Mainstream scheme. The calculations were carried out using a software written in the Ruby programming language.

START

INPUT THE DATA

t_k **: Elapsed days**
a_k **: Number of adult bees**
b_k **: Number of capped brood**



STEP 1

Discretize Eq. (S2) and solve a set of simultaneous equations in unknown u_k (number of newly-capped brood).

Eq.(S2) $\cdot\cdot\cdot$ $$b_k = \int_0^{Tp} u(tk-s)[p(tk-s)]^{\left(\frac{s}{Tp}\right)} ds$$

$$b_k = \sum_i ui \int_0^{Tp} [pi]^{\frac{s}{Tp}} ds$$

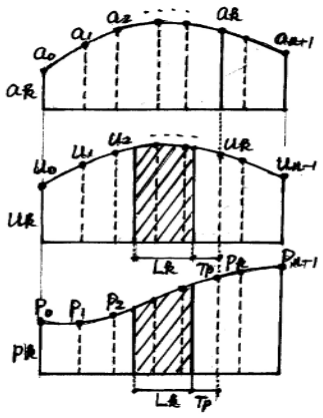
$$b_k = \mathrm{ui} \sum_i \int_0^{Tp} [pi]^{\frac{s}{Tp}} ds$$

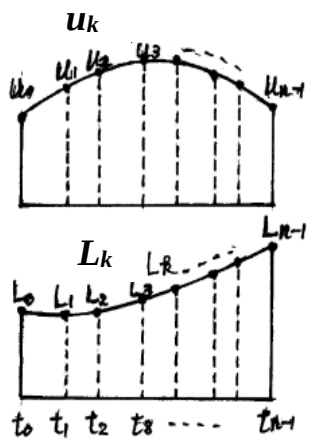
$$\mathrm{b} = [D] \cdot \mathrm{u}$$
$$\mathrm{u} = [D]^{-1} \cdot \mathrm{b}$$

STEP 2

Find unknown L_k in Eq.(S1) using u_k obtained by STEP 1 and input data of a_k and p_k by a bisection method.

Eq.(S1) $\cdot\cdot\cdot$ $$a_k = \int_{Tp}^{Tp+Lk} u(tk-s)p(tk-s)ds$$





PRINT THE DATA

t_k **,** a_k **,** b_k **,** p_k **,** u_k **,** L_k

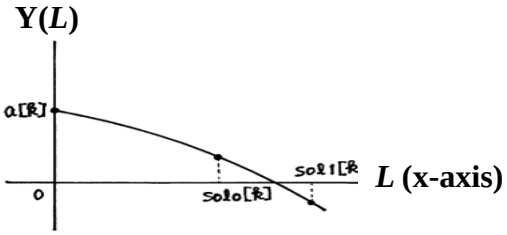
END

Supplementary Figure S4. Flowchart to find the apparent longevity, *L(t)*: Bisection method.

STEP 2
(Bisection method)

Create an evaluation function of Y(L) :

$$Y(L) = a[k] - \int_{Tp}^{Tp+L} u(tk-s)p(tk-s)ds$$



Set convergence criteria:
$x_\varepsilon = 10^{-4}$, $y_\varepsilon = 10^{-4}$

Execute the following process for the number of elements of $a[k]$

Increasing the value of L sequentially from zero in 0.5 increment, find the value of L where Y(L) is negative for the first time. Set the value of L at this time as sol-1[k] and then set previous value of L as sol-0[k].

Execute the following process for the number of elements of sol-1[k]

L_{t0} = sol-0[k], y_0 = Y(L_{t0})
L_{t1} = sol-1[k], y_1 = Y(L_{t1})

$|y_0| < y_\varepsilon$ or $|y_1| < y_\varepsilon$ or $|L_{t0} - L_{t1}| < x_\varepsilon$?

Yes

No

Calculate $L_{t2} = (L_{t0} + L_{t1})/2$ & y_2 = Y(L_{t2}) and then replace y_0 or y_1 with the same sign as y_2 with y_2 to make a new set of y_0 and y_1.

Add L_{t1} to the solution as $L_k = L_{t1}$.

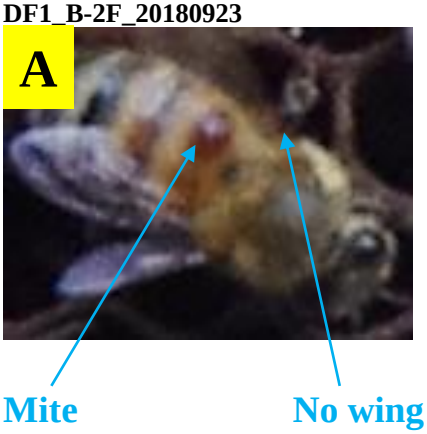
NEXT

Supplementary Figure S5. Images of mite-damaged bees.

The above images were obtained from the photographs of adult bees on comb-frame taken in the field experiments. Referring the above, we judged whether the adult bee was damaged by mites while enlarging the image. Several representative images of adult bees that appear to have been damaged by *Varroa* mites are shown. Each image is an example of a mite-damaged bee. **A:** A mite on the back of an adult bee (worker bee) whose wing is removed. **B:** Mites on the bee's leg and back. **C:** A mite on the head of a drone. **D:** The trace of a mite that fell out of the bee's back. **E:** Trace of a mite that fell out and a bee that has no wings. **F:** Traces of mites that fell out of the bee's back and a bee with no wing. **G:** Traces of a mite that fell out and a bee that seems to have been bitten by mites.

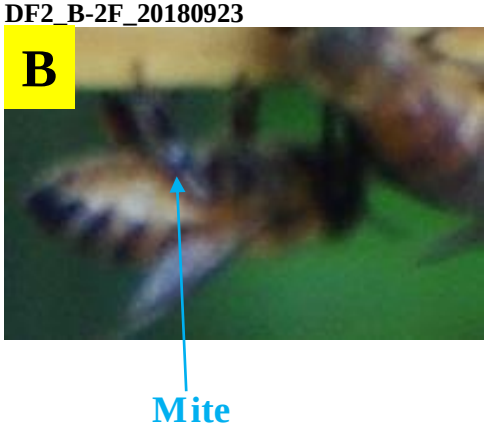
Mite on the bee's back

DF1_B-2F_20180923



Mite on the bee's leg & back

DF2_B-2F_20180923

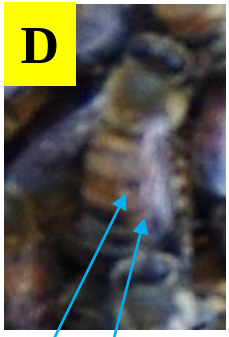


Mite on the head

DF2_B-2B_20180923

Traces of mites

DF2_B-2B_20180923

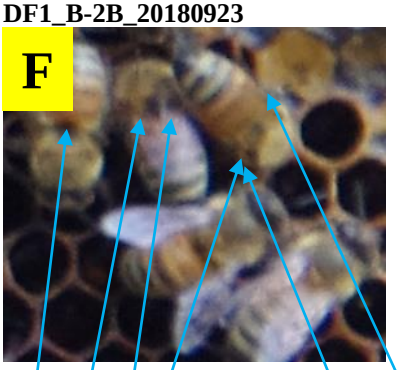


Traces of mites

DF2_B-4B_20180923

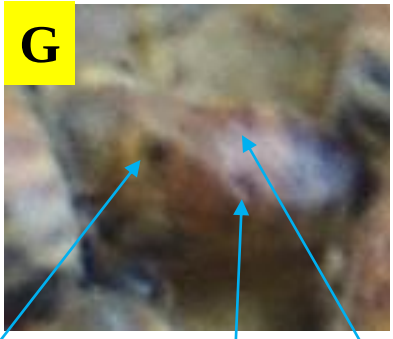
Traces of mites

DF1_B-2B_20180923



Traces of mites

DF2_B-2B_20180923a



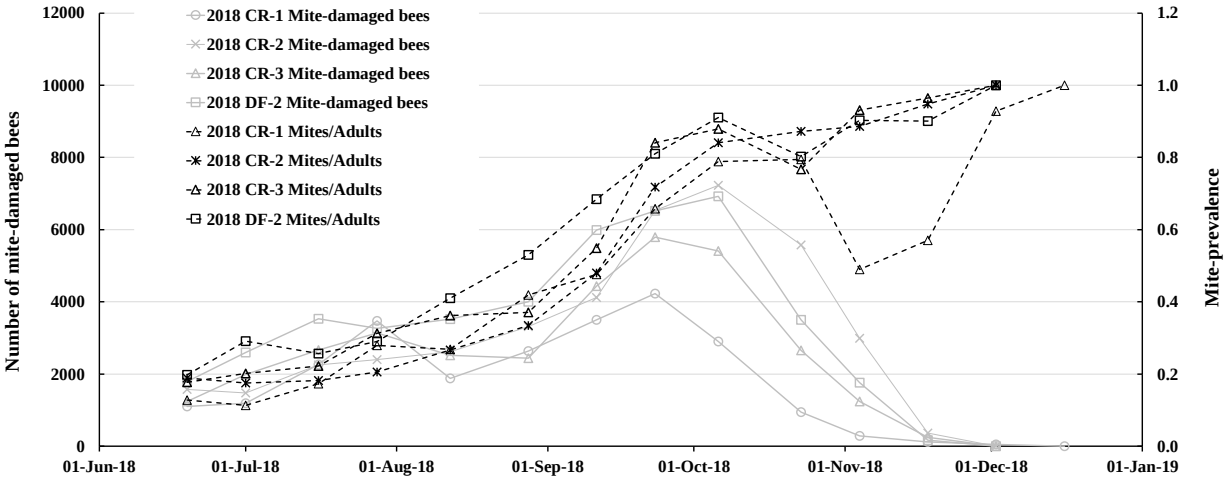
Supplementary Figure S6. Number of mite-damaged bees and mite-prevalence.

Details of these data in the field experiment conducted in 2018 are reported in the previous report (T. Yamada, 2020*)*. The mite-prevalence denotes the ratio of the number of damaged bees by *Varroa* mites (mite-damaged bees) to the total number of adult bees in a period between each measurement interval.

Mites/Adults denotes the mite-damaged bees /total bees (mite-prevalence).

2018 CR-1, 2018 CR-2 and 2018 CR-3 denote the control colony which is infested with *Varroa* mites.

2018 DF-1, 2018 DF-2 and 2018 DF-3 denote the colony being infested by *Varroa* mites and to which dinotefuran is administered via pollen paste.

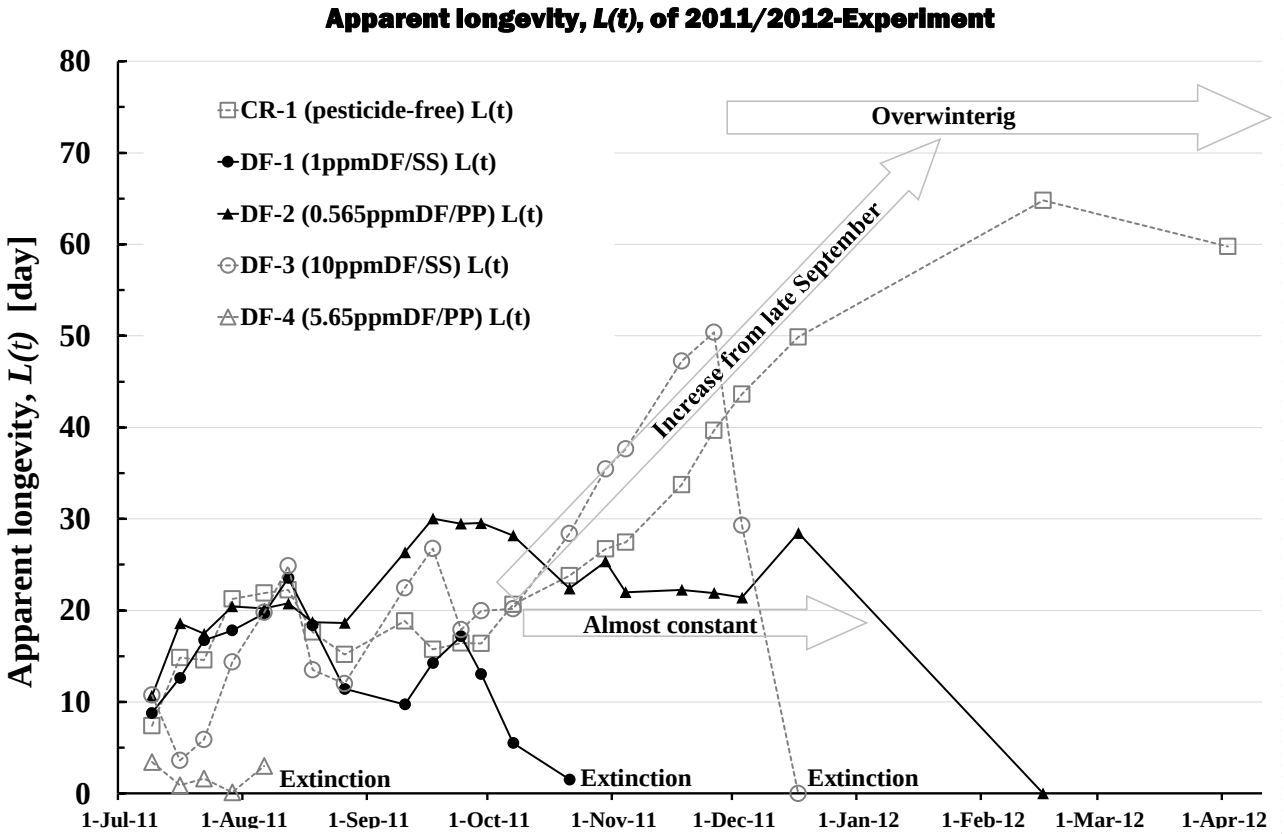

2018 CR-1 Mite-damaged bees
2018 CR-2 Mite-damaged bees
2018 CR-3 Mite-damaged bees
2018 DF-2 Mite-damaged bees
2018 CR-1 Mites/Adults
2018 CR-2 Mites/Adults
2018 CR-3 Mites/Adults
2018 DF-2 Mites/Adults
Number of mite-damaged bees
Mite-prevalence
12000
10000
8000
6000
4000
2000
0
1.2
1.0
0.8
0.6
0.4
0.2
0.0
01-Jun-18
01-Jul-18
01-Aug-18
01-Sep-18
01-Oct-18
01-Nov-18
01-Dec-18
01-Jan-19

Supplementary Figure S7. Apparent longevity of 2011/2012-Experiment.

CR-1 (pesticide-free) L(t): Apparent longevity of control colony (**CR-1**) to which pesticide is not administered. **DF-1 (1ppmDF/SS) L(t):** Apparent longevity of experimental colony (**DF-1**) to which dinotefuran (a kind of neonicotinoids) of 1 ppm is administered via sugar syrup. **DF-2 (0.565ppmDF/PP) L(t):** Apparent longevity of experimental colony (**DF-2**) to which dinotefuran 0.565 ppm is administered via pollen paste. **DF-3 (10ppmDF/SS) L(t):** Apparent longevity of experimental colony (**DF-3**) to which dinotefuran of 10 ppm is administered via sugar syrup. **DF-4 (5.65ppmDF/PP) L(t):** Apparent longevity of experimental colony (**DF-4**) to which dinotefuran 5.65 ppm is administered via pollen paste. All colonies are almost mite-free.

The control colony, **CR-1**, and experimental colony, **DF-3**, begin to increase the apparent longevity from late September, as if they already have known that winter is approaching. **CR-1** continues to increase during wintering, but **DF-3** have become extinct soon after wintering. The extinction of **DF-3** is thought to be due to the fact that the colony has been already weakened by dinotefuran before wintering, as evidenced by the fact that other experimental colonies, **DF-1** and **DF-4**, became extinct before wintering. **DF-1** and **DF-3** are both bee colonies that received dinotefuran via sugar syrup, but **DF-1** with a low concentration of dinotefuran became extinct earlier than **DF-3** with a high concentration of dinotefuran (see the previous paper (T. Yamada et al., 2018a) for details).

On the other hand, as if another experimental colony, **DF-2**, does not notice that winter is approaching, its apparent longevity does not begin to increase from late September, and the longevity remains almost constant even into wintering. The almost constant longevity changes through the year for a bee colony are quite different from general seasonal longevity changes. This unusual change cannot be explained by the normal physiology of bees. The difference between the seasonal changes in the apparent longevity of DF-2 and that of DF-3 as described above is thought to be due to the difference in the food (vehicles) via which dinotefuran is administer. The mechanism by which this difference occurs is discussed in this paper.

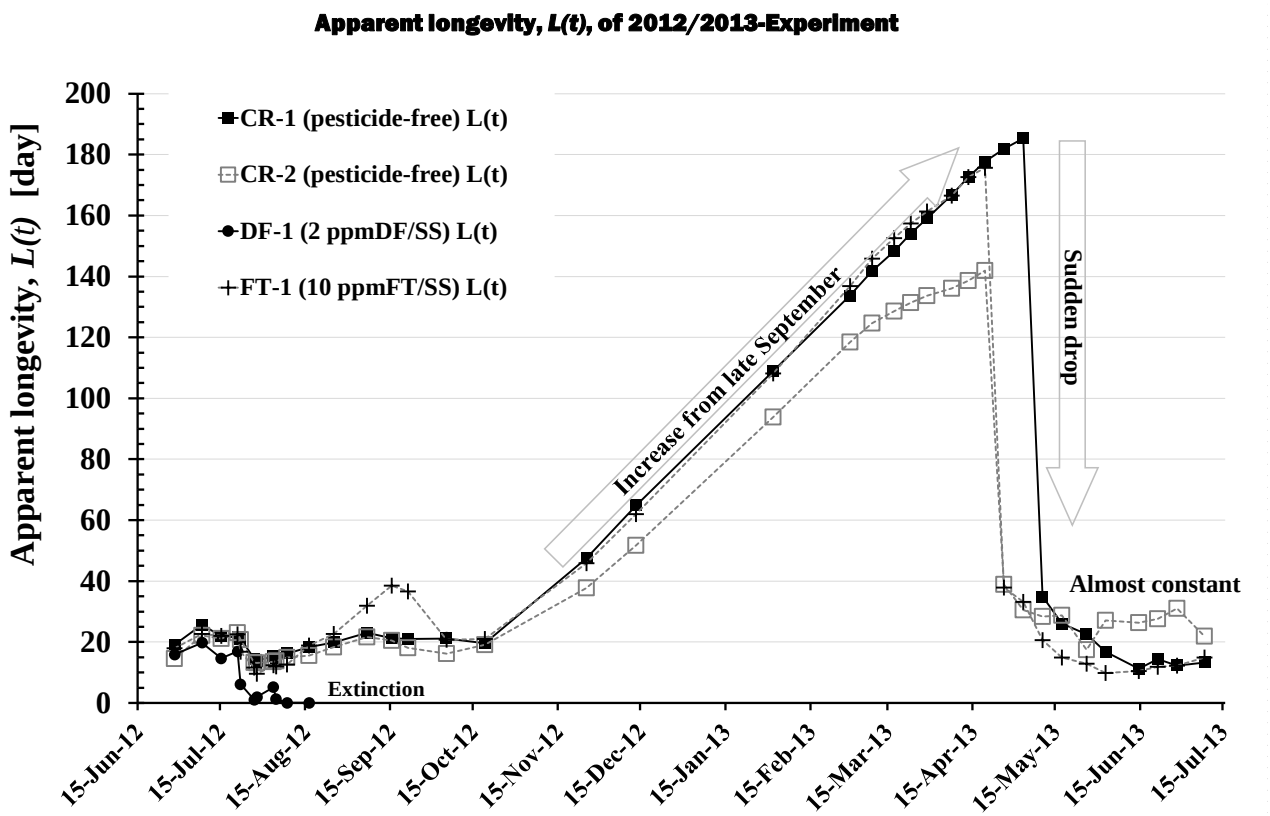

Apparent longevity, L(t), of 2011/2012-Experiment
Apparent longevity, L(t) [day]
80
70
60
50
40
30
20
10
0
CR-1 (pesticide-free) L(t)
DF-1 (1ppmDF/SS) L(t)
DF-2 (0.565ppmDF/PP) L(t)
DF-3 (10ppmDF/SS) L(t)
DF-4 (5.65ppmDF/PP) L(t)
Overwinterig
Increase from late September
Almost constant
Extinction
Extinction
Extinction
1-Jul-11
1-Aug-11
1-Sep-11
1-Oct-11
1-Nov-11
1-Dec-11
1-Jan-12
1-Feb-12
1-Mar-12
1-Apr-12

Supplementary Figure S8. Apparent longevity of 2012/2013-Experiment.

CR-1 (pesticide-free) L(t) and CR-2 (pesticide-free) L(t): Apparent longevity of control colonies (**CR-1**, **CR-2**) to which pesticide is not administered. **DF-1 (2ppmDF/SS) L(t):** Apparent longevity of experimental colony (**DF-1**) to which dinotefuran of 2 ppm is administered via sugar syrup. **FT-1 (10ppmFT/SS) L(t):** Apparent longevity of experimental colony (**FT-1**) to which fenitrothion of 10 ppm is administered via sugar syrup. All colonies are almost mite-free.

The experimental colony **DF-1** became extinct early. Pesticide-free control colonies **CR-1** and **CR-2**, as well as fenitrothion (organophosphate) containing colonies **FT-1**, exhibit general seasonal longevity changes. That is, their longevities are short and almost constant from the end of wintering to late September, then begins to extend from late September, continues to extend until just before the end of wintering, and when wintering is over, it suddenly drops to a short level, after which it stabilizes.

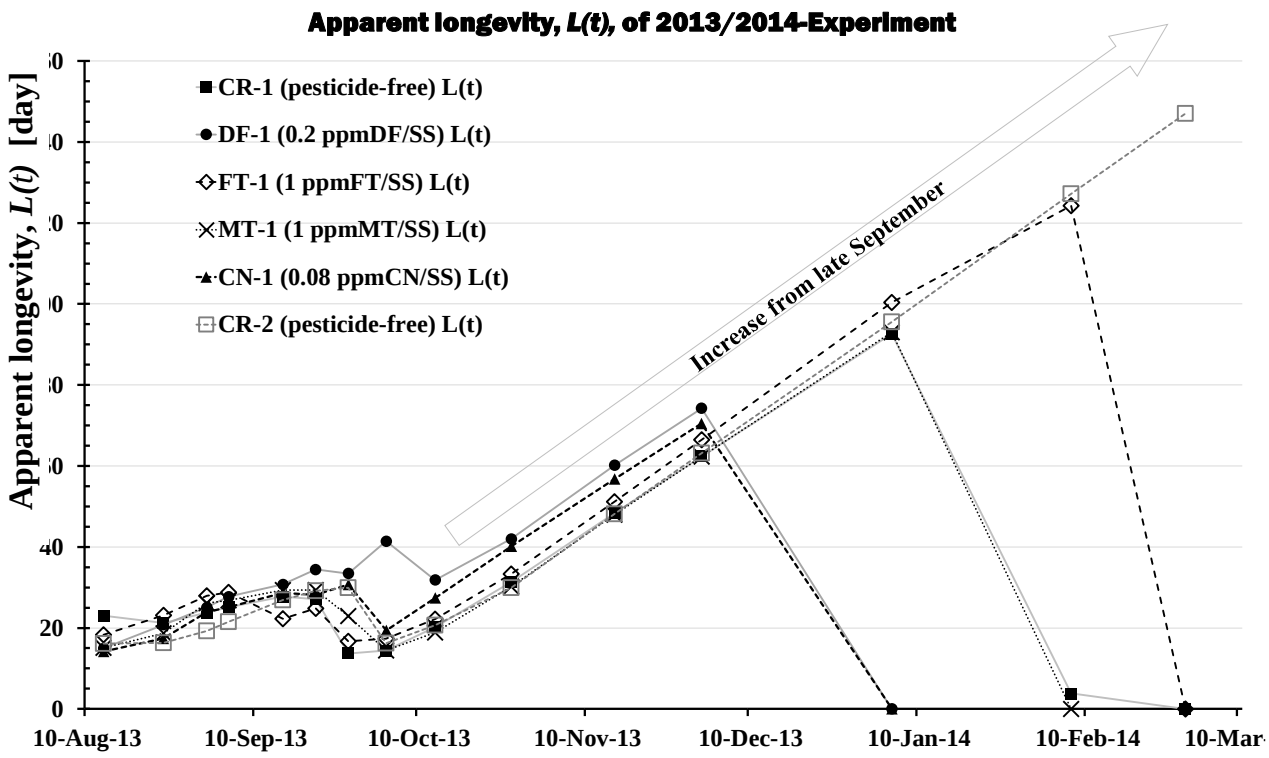
It is noteworthy that **FT-1** where is administered fenitrothion of an amount having the same acute insecticidal activities as 2 ppm dinotefuran, succeeded in wintering, just like the control groups (**CR-1** and **CR-2**). The difference between the early extinction of **DF-1** and the successful wintering of **FT-1** is probably due to the difference in the length of the residual effect period. For details, please refer to the previous report (T. Yamada et al., 2018b).



Supplementary Figure S9. Apparent longevity of 2013/2014-Experiment.

CR-1 (pesticide-free) L(t) and CR-2 (pesticide-free) L(t): Apparent longevity of control colonies (**CR-1**, **CR-2**) to which pesticide is not administered. **DF-1 (0.2 ppmDF/SS) L(t):** Apparent longevity of experimental colony (**DF-1**) to which dinotefuran of 0.2 ppm is administered via sugar syrup. **FT-1 (1 ppmFT/SS) L(t):** Apparent longevity of experimental colony (**FT-1**) to which fenitrothion of 1 ppm is administered via sugar syrup. **MT-1 (1 ppmMT/SS) L(t):** Apparent longevity of experimental colony (**MT-1**) to which malathion of 1 ppm is administered via sugar syrup. **CN-1 (1 ppmCN/SS) L(t):** Apparent longevity of experimental colony (**CN-1**) to which clothianidin of 0.08 ppm is administered via sugar syrup. All colonies are almost mite-free.

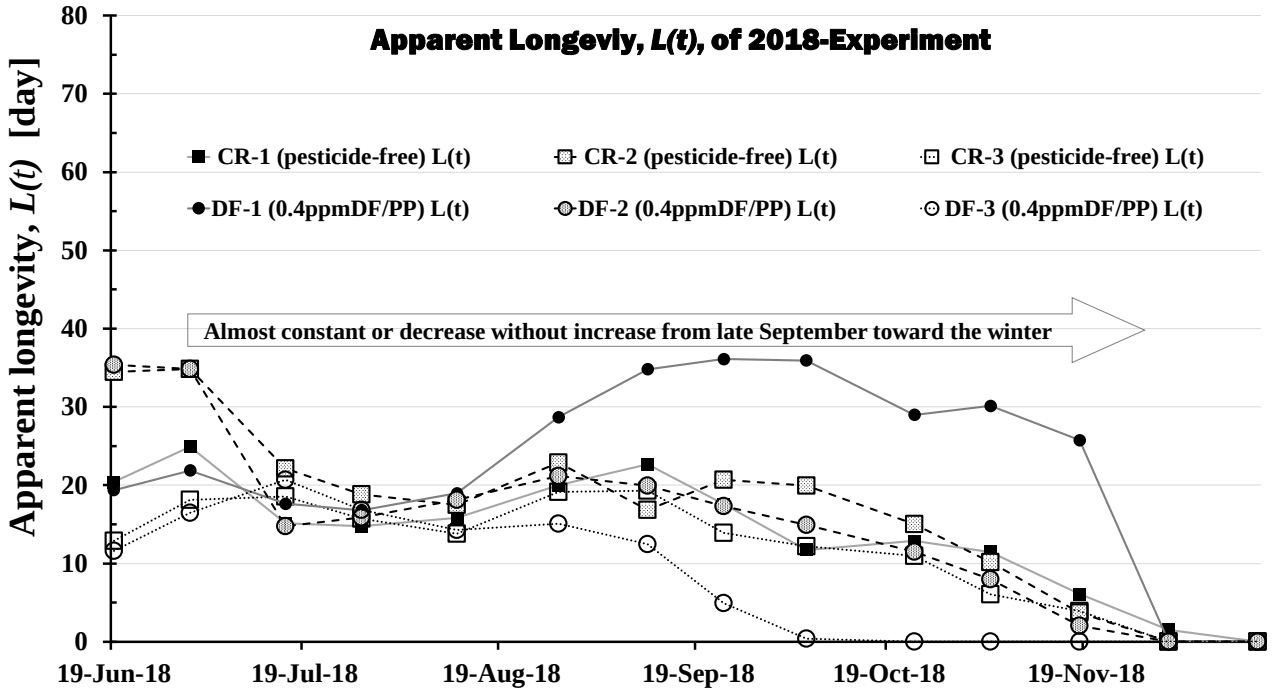
The apparent longevity of all colonies (**CR-1**, **CR-2**, **DF-1**, **FT-1**, **MT-1**, **CN-1**) changed in the same way; it held almost constant at a short level from the start of experiment till late September, then began to extend toward winter, and continued to extend during wintering, but all colonies other than **CR-2** became extinct during wintering.



Supplementary Figure S10. Apparent longevity of 2018-Experiment.

CR-1 (pesticide-free) L(t), CR-2 (pesticide-free) L(t) and CR-3 (pesticide-free) L(t): Apparent longevity of control colonies (**CR-1**, **CR-2**, **CR-3**) to which pesticide is not administered. **DF-1 (0.4 ppmDF/PP) L(t), DF-2 (0.4 ppmDF/PP) L(t) and DF-3 (0.4 ppmDF/PP) L(t):** Apparent longevity of experimental colonies (**DF-1**, **DF-2**, **DF-3**) to which dinotefuran of 0.4 ppm is administered via pollen paste. All colonies were infested with mites (T. Yamada, 2020).

The apparent longevities of all colonies in this field experiment are not the typical seasonal changes that begin to extend from late September and continues until the end of wintering, but has an unusual seasonal change that is almost constant or slightly shorter towards winter. Interestingly, the apparent longevity of all colonies in this experiment is, roughly speaking, fairly constant throughout the year, with little seasonal changes in apparent longevity. This is similar to the seasonal changes in the apparent longevity of bee colonies fed dinotefuran (neonicotinoid pesticides) via pollen paste (T. Yamada et al., 2018a).



3 Supplementary Tables (Tables.S1 to S7)

Supplementary Table S1. Outline of pesticide intake in three field experiments.

Supplementary Table S2. The essentials of experimental conditions and results for four long-term field experiments conducted in Shika, Japan.

Supplementary Table S3. Apparent longevity of a honeybee colony in the 2011/2012 experiment at the eclosion rate of 0.9.

Supplementary Table S4. Apparent longevity of a honeybee colony in the 2012/2013 experiment at the eclosion rate of 0.9.

Supplementary Table S5. Apparent longevity of a honeybee colony in the 2013/2014 experiment at the eclosion rate of 0.9.

Supplementary Table S6. Apparent longevity, $L(t)$, of a honeybee colony in the 2018 experiment at the eclosion rate of 0.9.

Supplementary Table S7. Number of mite-damaged bees and mite-prevalence in 2018 and 2011/2012 experiments.

Supplementary Table S1. Outline of pesticide intake in three field experiments.

Location of experimental site: mid-west Japan (Shika-machi) (Latitude 37°1'9"N; Longitude 136°46'14"N).

	Initial number of adult bees	Initial number of capped brood	Administration period of pesticide	Total intake of pesticide per colony during administration [mg/colony]	Total number of adult bees during administration	Total intake of pesticide per bee during administration [ng/bee]	Extinction or survival
2011/2012 CR-1 (pesticide-free)[25]				0		0	Survival
2011/2012 DF 1ppm/syrup[25]	2965	4263	9-Jul-11 to 21-Oct-11	4.208	13543	310.7	Extinction
2011/2012 DF-0.565ppm/pollen[25]	2158	2556	9-Jul-11 to 3-Dec-11	1.8692	30779	60.7	Extinction
2011/2012 DF 10ppm/syrup[25]	3296	3880	9-Jul-11 to 16-Jul-11	1.9	6344	290.3	Extinction
2011/2012 DF 5.65 ppm/pollen[25]	1659	6993	9-Jul-11 to 16-Jul-11	0.5257	3078	65.1	Extinction
2012/2013 CR-1 (pesticide-free)[26]				0		0	Survival
2012/2013 CR-2 (pesticide-free)[26]				0		0	Survival
2012/2013 DF 2ppm/syrup[26]	9173	9442	21-Jul-12 to 16-Aug-12	1.55	16524	93.8	Extinction
2012/2013 FT 10ppm/syrup[26]	8943	8732	21-Jul-12 to 16-Aug-12	17.97	19792	862.5	Survival
2013/2014 CR-1 (pesticide-free)[27]			0	0		0	Extinction
2013/2014 CR-2 (pesticide-free)[27]			0	0		0	Survival
2013/2014 DF 0.2ppm/syrup[27]	6017	2214	5-Sep-13 to 1-Dec-13	0.514	8612	56.7	Extinction
2013/2014 CN 0.08ppm/syrup[27]	7293	1506	5-Sep-13 to 1-Dec-13	0.4016	10130	39.6	Extinction
2013/2014 FT 1ppm/syrup[27]	10296	3232	5-Sep-13 to 28-Feb-14	3.86	19585	197.1	Extinction
2013/2014 MT 1ppm/syrup[27]	7676	1396	5-Sep-13 to 7-Feb-14	6.75	15724	429.3	Extinction

Supplementary Table S2. The essentials of experimental conditions and results for four long-term field experiments conducted in Shika, Japan.

Experiment name (Experimetal period)	**2011/2012 experiment[25)]** From July 9, 2011 to April 2, 2012 (268 days)	**2012/2013 experiment[26)]** From June 28, 2012 to July 26, 2013 (393 days)	**2013/2014 experiment[27)]** From Aug. 13, 2013 to Feb. 28, 2014 (199 days)	**2018 experiment[28)]** From Jul. 19, 2018 to Dec. 16, 2018 (180 days)
Object of Study	To investigate the difference in the long-term impact on a bee colony between toxic sugar syrup (honey) as an energy source and toxic pollen pase (bee bread) as a protein source which are exposed to a neonicotinoid	To investigate the difference in the long-term impact on a bee colony between a neonicotinoid and a organophosphate which are administered through sugar syrup	To investigate the difference in the long-term impact on a bee colony between a neonicotinoid and a organophosphate which are administered through sugar syrup under lower concentrations than in the previous work	To investigate the seasonal changes in colony size and mite-prevalence in a honeybee colony exposed to dinotefuran via pollen paste and infested with *Varroa* mites
Circumstances around Experimental Site				
Experimental site (latitude and longitude)	Shika (mid-west Japan) (37°1'9" N, 136°46'14" E, 43m above sea level)	Same as the left	Same as the left	Shika (mid-west Japan) (37 °2 '35" N, 136 °45 '38" E, 70m above sea level)
Limitation of honeybee activities	Honeybees can freely forage about for food in a hive or in fields	Same as the left	Same as the left	Same as the left
Environmental effects of pesticides other than administered pesticides	A pesticide-free watering place & a pesticide-free field of flowers in the apiary	Same as the left	Same as the left	Same as the left
Aerial-crop-dusting farmland near experimental site	Nothing	Nothing	Nothing	Nothing
Seasonal changes	Distinct	Distinct	Distinct	Distinct
Experimental Conditions				
Materials	Vehicle : sugar syrup; pollen paste. Pesticide: dinotefuran	Vehicle: sugar syrup; pollen paste. Pesticide: dinotefuran;fenitrothion	Vehicle: sugar syrup; pollen paste. Pesticide: dinotefuran; clothianidin; fenitrothion; malathion	Vehicle: sugar syrup; pollen paste. Pesticide: dinotefuran
Initial numbers of apiaries, colonies and combs (frames)	One apiary (private), five colonies per apiary, three combs per colony (one colony for each same conditions)	One apiary (private), four colonies per apiary, three combs per colony (one colony for each same conditions except 2 controls)	One apiary (private), six colonies per apiary, three combs per colony (one colony for each same conditions except 2 controls)	One apiary (private), six colonies per apiary, three combs per colony (3 control colonies & 3 dinotefuran-exposed colonies)
Concentration of pesticide	Dinotefuran: 1 & 10 ppm in sugar syrup, 0.565 & 5.65 in pollen paste	Dinotefuran: 2 ppm in sugar syrup. Fenitrothion: 10 ppm in sugar syrup	Dinotefuran: 0.2 ppm in sugar syrup. Clothianidin: 0.08 ppm in sugar syrup. Fenitrothion & Malathion: 1 ppm in sugar syrup	Dinotefuran: 0.4 ppm in pollen paste
Initial number of bees per colony	1700 to 3400 bees (accurately counted from photos)	5600 to 7100 bees (accurately counted on photos)	5400 to 7600 bees (accurately counted on photos)	7000 to 9000 bees (accurately counted on photos)
Initial number of capped brood per colony	2600 to 6100 capped brood (accurately counted from photos)	4000 to 5700 capped brood (accurately counted from photos)	4200 to 7600 capped brood (accurately counted from photos)	3100 to 6900 capped brood (accurately counted from photos)
Origin of a queen	Unknown in detail (Apis mellifera) Bee colonies purchased from a bee farm	Same as the left	Same as the left	Queen bees in sister relationship
Experimental Methods				
Interval of experiment	About one-week interval	About one-week or two-weeks interval	Same as left	About two-weeks inter
Administration period of pesticide	From July 9, 2011 to the colony extinction, or to December 3 (147 days)	From July 21, 2012 to the colony extinction, or to August 16 (26 days)	From September 5, 2013 to the colony extinction, or to December 1 (87 days)	From July 1, 2018 to the colony extinction
Starting time of each observation	Just after dawn if possible (before bees go out to forage)	Same as the left	Same as the left	Same as the left
Vehicle to administer a pesticide	Either sugar Syrup or pollen paste	Sugar syrup	Sugar syrup	pollen paste
Administration method of pesticide	A pesticide was dissolved in sugar syrup or pollen was kneaded with toxic sugar syrup containing the pesticide. Either toxic sugar syrup or toxic pollen paste was fed into a hive	A pesticide was dissolved in sugar syrup with toxic sugar syrup containing the pesticide. Only toxic sugar syrup was fed into a hive.	A pesticide was dissolved in sugar syrup with toxic sugar syrup containing the pesticide. Only toxic sugar syrup was continuously fed into a hive with an auto-feeding system composed of 10 L (14 kg syrup) container	Pollen paste was made while pollen was being kneaded with toxic sugar syrup containing the pesticide. Toxic pollen paste was fed into a hive.
Counting method of the number of adult bees	Directly counted with accuracy from photos of combs with bees and bees left in a hive after every comb was removed from it with the help of a automatic counting software	Same as the left (auxiliary automatic counting software has been improved)	Same as the left (auxiliary automatic counting software has been improved)	Same as the left (auxiliary automatic counting software has been improved)
Counting method of the number of capped brood	Acurately counted from photos of combs without bees after shaking the bees off each comb with the help of a automatic counting software	Same as the left (auxiliary automatic counting software has been improved)	Same as the left (auxiliary automatic counting software has been improved)	Same as the left (auxiliary automatic counting software has been improved)
Total intake of pesticide per colony	Calculated from the sugar syrup or pollen paste with pesticide consumed by honeybees.	Calculated from the sugar syrup with pesticide consumed by honeybees	Same as the left	Same as the left
Estimation of the intake of pesticide per bee	Estimated from dividing the total intake of pesticide per colony by the total number of initial & newly-emerged honeybees	Same as the left	Same as the left	Same as the left
Counting method of number of dead bees	Dead bees were accurately counted one by one inside and outside a hive which was placed on a large tray	Same as the left	Same as the left	Same as the left
Confirmation and record methods of a queen	A photographic record of the exsistence of a queen in each colony	Same as the left	Same as the left	Same as the left
Publications of Research Results (Title, Journal, Short Abstract)				
Experimental Results	A clear difference in the impact on honeybee (*Apis mellifera*) colony between the two vehicles of sugar syrup and pollen paste	Difference between the impact of the neonicotinoid dinotefuran and organophosphate fenitrothion on a bee colony in a long-term field experiment: An evidence	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)	Seasonal changes in the size and mite-prevalence of a bee colony exposed to dinotefuran via pollen paste and damaged by Varroa mites
	T. Yamada et al. (2018). *J. Biol. Ser.* 1(3): 084-107.	Yamada et al. (2018). *J. Biol. Ser.* 1(3): 108-137	T. Yamada et al. (2018). *J. Biol. Ser.* 1(4): 187-207.	Yamada T. (2020). *J. Diet. Res. Nutr.* 7(1): 002.
	This study investigates the difference in the impact of the neonicotinoid pesticide dinotefuran on honeybee colony between the two vehicles of sugar syrup and pollen paste. A distinct difference was observed between the two vehicles: The per-bee intake of dinotefuran administered through pollen paste as a vehicle until colony extinction was roughly one-fifth of the per-bee intake administered through sugar syrup, independently of dinotefuran concentrations. This difference can be attributed to the dissimilarity in strength of the impact on honeybee colony between worker bees which preferentially take sugar syrup (honey) to pollen paste and a queen bee and brood (larvae) which take pollen paste (bee bread) in preference to sugar syrup as a result of the long-lasting toxicity of dinotefuran. This suggests that pollen as a protein source contaminated by neonicotinoid pesticides can cause deeper adverse effect on a honeybee colony than honey as an energy source.	We conducted a long-term field experiment and found different impacts on honeybee colonies in an apiary between the neonicotinoid dinotefuran and the organophosphate fenitrothion. The colony where dinotefran was administered (dinotefuran colony) became extinct in the administration period of 26 days, while the colony where fenitrothion was administered (fenitrothion colony) survived long after the same administration period. The fenitrothion colony succeeded in overwintering and staying alive for more than 293 days after administration. We speculate that colonies exposed to dinotefuran hardly recover from the damage because dinotefuran has a much longer persistent ability than fenitrothion and toxic foods stored in cells over a prolonged period of time can affect a colony.	In this paper the differences are investigated between neonicotinoids (dinotefuran, clothianidin) and organophosphates (fenitrothion, malathion) on a realistic pesticide-concentration level in the natural environment surrounding an apiary in Japan. It has been shown that neonicotinoids can much more rapidly weaken the colony where it was administered than organophosphates and organophosphates can be rapidly degraded in honey stored because of their high degradability. These results roughly reproduced the findings in our previous experiment. Neonicotinoid-concentration in residual honey in comb-cells were lower than those in sugar syrup fed to each colony were detected but organophosphates were hardly detected. We inferred that obscure massive colony losses in winter can be probably caused by toxic food with a long-term persistent pesticide such as a neonicotinoid stored in combs during overwintering after the weakening of the colony due to the ingestion of toxic nectar, pollen and water under the natural circumstances contaminated by long-persistent pesticides such as neonicotinoids just before	In this study, the impact of dinotefuran (DF) administered via pollen paste to a bee colony damaged by Varroa mites was investigated. Seasonal changes in the number of adult bees (those capped brood) and the mite-prevalence among adult bees were measured through a field experiment over 180 days. It was found that the bee colony collapsed under the intake of a smaller amount of DF due to the synergistic effect of DF and mite-damage. Because the daily pesticide-free sugar-syrup intake per bee in the DF-exposed colony administered via pollen paste was greater than that of the control colony, DF may have an appetite-promoting effect. Since the consumption of DF-containing pollen paste per bee per day showed almost no difference between all colonies, DF seems to have no repellent effect. Mite-prevalence continued to rise and became almost 100% near the time of colony extinction. The inner-temperature-fluctuation-range of the hive-box (Ti) was smaller than that of the ambient temperature (Ta). The inner-temperature-fluctuation-range of the DF-exposed-colony hive-box was larger
Estimated results of apparent longevity	A mathematical model to estimate the seasonal change in apparent longevity of bee colony			
	Y. Yamada et al. (2019). *Scientific Reports*, volume 9, Article number: 4102 (2019)			
	We have proposed a new mathematical model to estimate the apparent longevity defined in the upper limit of an integral equation. The apparent longevity can be determined only from the numbers of adult bees and capped brood. By applying the mathematical model to a honeybee colony in Japan, seasonal changes in apparent longevity were estimated in three long-term field experiments. Three apparent longevities showed very similar season-changes to one another, increasing from early autumn, reaching a maximum at the end of overwintering and falling approximately plumb down after overwintering. (This study was performed using only the control colonies.)			
	Damage to larvae causes seasonal changes in honeybee longevity to go crazy			
	T.Yamada and Y. Yamada., preparing paper submission (this work)			
	The followings were clarified from the honeybee longevity estimated by the mathematical model using the numbers of adult bees and capped brood obtained from three long-term field experiments. The longevities of the mite-free control colony and the mite-free colony where a pesticide is administered via sugar syrup show common seasonal changes, which continue to increase from the end of September to the end of overwintering and drop rapidly just after overwintering, though the ingestion of a pesticide via sugar syrup shortens the honeybee longevity. However, the longevity of the mite-infested colony and that of the mite-free colony where a pesticide is administered via pollen paste show abnormal seasonal changes in longevity that cannot increase since late September. Such abnormal seasonal changes may be caused by biological rhythm disorders due to damages by toxic pollen paste and parasitic mites during the brood stage when seasonal sensory functions and organs are created. We infer that such damages to larvae and pupae can disrupt bees' internal clocks and make honeybees undetectable of the arrival of winter, and make the colony fail in overwintering because the longevity does not extend due to abnormal seasonal changes in longevity. (This study was performed using all the colonies other than those that collapsed before mid-November)			

Supplementary Table S3. Apparent longevity of a honeybee colony in the 2011/2012 experiment at the eclosion rate of 0.9.

This table shows the data source of the apparent longevity in Figure S7, which were estimated using the field experimental results conducted in 2011 to 2012. *a(t)* and *b(t)* denote the number of adult bees and the number of capped brood obtained from the field experiment conducted from 2011 to 2012, respectively (T. Yamada et al., 2018a). *L(t)* is the apparent longevity estimated using *a(t)* and *b(t)* by the mathematical model previously proposed (*1*). "CR-1" denotes the control colony where mites are almost absent and a pesticide is not administered. "DF 1ppm/SS" and "DF 10ppm/SS" denote the colonies where the neonicotinoid dinotefuran of 1 ppm and 10 ppm are administered via sugar syrup, respectively. "DF 0.565ppm/PP" and "DF 5.65ppm/PP" denote the colonies where the neonicotinoid dinotefuran of 0.565 ppm and 5.65 are administered via pollen paste, respectively. **Note:** A pesticide-administration period is from the morning of July 9 in 2011 until a colony becomes extinct, but until the morning of December 3 when a colony survives to the winter (T. Yamada et al., 2018a).

Date	Elapsed days	CR-1			DF 1ppm/SS			DF 0.565ppm/PP			DF 10ppm/SS			DF 5.65ppm/PP		
		a(t)	*b(t)*	*L(t)*	*a(t)*	*b(t)*	*L(t)*	*a(t)*	*b(t)*	*L(t)*	*a(t)*	*b(t)*	*L(t)*	*a(t)*	*b(t)*	*L(t)*
9-Jul-11	0	3392	5819	7.38	2965	4263	8.80	2158	2556	10.68	3295	3880	10.75	1659	6093	3.45
16-Jul-11	7	6827	3644	14.85	4257	1287	12.64	3755	2377	18.59	1102	986	3.59	430	2865	0.89
22-Jul-11	13	6399	4692	14.59	5233	1655	16.75	3493	3207	17.45	1407	232	5.88	330	1165	1.62
29-Jul-11	20	7737	4861	21.27	3131	1034	17.82	4025	2743	20.45	1451	939	14.38	17	790	0.12
6-Aug-11	28	7893	7179	21.89	2393	1591	19.66	4372	3480	20.21	1384	2370	19.78	1	729	3.02
12-Aug-11	34	7675	8390	22.26	1762	2288	23.55	4513	3217	20.77	1752	3217	24.85			
18-Aug-11	40	8873	6125	17.63	2298	1137	18.38	4829	1933	18.72	2257	946	13.50			
26-Aug-11	48	9327	5797	15.17	1623	1757	11.42	4276	2099	18.63	2271	913	11.99			
10-Sep-11	63	9249	8803	18.82	1253	318	9.73	4620	1343	26.35	2096	545	22.46			
17-Sep-11	70	9762	8327	15.75	997	341	14.26	4241	1546	30.04	1760	707	26.76			
24-Sep-11	77	11252	7034	16.44	462	453	17.18	3862	2693	29.47	908	772	17.90			
29-Sep-11	82	10736	5810	16.37	342	230	13.06	3792	2445	29.54	996	505	19.94			
7-Oct-11	90	12015	6100	20.65	169	109	5.51	4232	2082	28.18	1076	12	20.19			
21-Oct-11	104	11253	4100	23.79	0	45	1.53	4225	1935	22.40	860	220	28.40			
30-Oct-11	113	10958	4300	26.73				4044	2105	25.35	895	147	35.46			
4-Nov-11	118	10654	3900	27.44				3675	2665	21.99	790	245	37.66			
18-Nov-11	132	11303	3430	33.75				4022	1720	22.25	779	160	47.23			
26-Nov-11	140	12390	1750	39.66				3806	1034	21.91	630	48	50.39			
3-Dec-11	148	12109	2038	43.64				2992	840	21.41	393	21	29.30			
17-Dec-11	162	11811	2038	49.87				2433	157	28.47	0	0	0.00			
16-Feb-12	223	10514	2038	64.81				0	0	0.00						
2-Apr-12	269	9622	2038	59.75												

Supplementary Table S4. Apparent longevity of a honeybee colony in the 2012/2013 experiment at the eclosion rate of 0.9.

This table shows the data source of the apparent longevity in Figure S8, which were estimated using the field experimental results conducted in 2012 to 2013. *a(t)* and *b(t)* denote the number of adult bees and the number of capped brood obtained from the field experiment conducted from 2012 to 2013, respectively (T. Yamada et al., 2018b). *L(t)* is the apparent longevity estimated using *a(t)* and *b(t)* by the mathematical model previously proposed (*1*). "CR-1" and "CR-2" denote the control colonies where a pesticide is not administered, respectively. "DF 2ppm/SS" denotes the colonies where the neonicotinoid dinotefuran of 2 ppm is administered via sugar syrup. "FT 10ppm/SS" denotes the colony where the organophosphate fenitrothion of 10 ppm is administered via sugar syrup. Mites did not little exist in all colonies. **Note:** A pesticide-administration period is from the morning of July 21 in 2012 until the morning of August 16 (T. Yamada et al., 2018b).

Date	Elapsed days	CR-1			CR-2			DF 2ppm/SS			FT 10ppm/SS		
		a(t)	*b(t)*	*L(t)*	*a(t)*	*b(t)*	*L(t)*	*a(t)*	*b(t)*	*L(t)*	*a(t)*	*b(t)*	*L(t)*
28-Jun-12	0	7136	4746	19.03	5832	5094	14.49	7119	5679	15.86	5690	4012	17.95
8-Jul-12	10	9621	5806	25.65	8917	4710	22.15	8877	6446	19.78	7157	5286	22.58
15-Jul-12	17	8695	10215	21.94	8265	11143	21.07	6878	13267	14.61	7565	9619	22.06
21-Jul-12	23	9647	10254	21.53	9665	11301	23.06	9173	9442	16.91	8943	8732	22.56
22-Jul-12	24	10136	10210	21.12	9558	10967	20.76	4885	8834	6.11	7750	8694	16.76
27-Jul-12	29	10633	10617	14.51	10770	10329	13.24	1434	4548	1.03	8721	6563	11.57
28-Jul-12	30	10391	10858	13.79	10901	10581	13.15	1313	3891	1.90	7786	6389	9.52
3-Aug-12	36	12083	10000	15.41	11939	10025	13.52	1049	1131	5.15	8289	3390	12.31
4-Aug-12	37	12389	9687	14.88	12041	10269	13.70	771	840	1.22	7559	2901	11.96
8-Aug-12	41	14065	7154	16.23	12978	8472	14.86	33	208	0.05	6625	1352	12.62
16-Aug-12	49	13371	6111	18.39	12207	5977	15.60	0	0	0.00	5961	607	18.86
25-Aug-12	58	11961	6014	19.88	10997	6684	18.37				4467	918	22.64
6-Sep-12	70	11165	8783	23.00	11582	8126	21.68				3534	3406	31.86
15-Sep-12	79	11980	5531	21.31	11825	7135	20.47				4576	4187	38.45
21-Sep-12	85	12166	6086	21.00	11025	9202	18.04				5859	4119	36.60
5-Oct-12	99	10715	7615	21.14	10510	5679	16.13				6593	4648	20.80
19-Oct-12	113	11726	7280	19.59	10038	6628	19.03				7326	4713	20.95
25-Nov-12	150	13255	36	47.53	12477	2937	37.74				7755	10	45.86
13-Dec-12	168	12858	0	64.81	13316	305	51.75				7080	0	62.00
1-Feb-13	218	9306	0	108.91	9421	0	93.76				5652	0	108.11
1-Mar-13	246	7464	17	133.75	8426	0	118.44				5957	26	136.85
9-Mar-13	254	7512	497	141.82	8017	660	124.67				6358	619	145.90
17-Mar-13	262	6862	1691	148.28	7372	2419	128.57				6152	2329	152.54
23-Mar-13	268	7312	2431	154.08	7416	3624	131.34				6470	3217	157.36
29-Mar-13	274	7720	4097	159.04	8018	5165	133.66				7143	4994	161.37
7-Apr-13	283	9518	7326	166.80	9833	8414	136.13				8797	7053	166.52
13-Apr-13	289	12523	10166	172.59	12594	11211	138.63				12038	8670	172.62
19-Apr-13	295	15677	11324	177.64	16221	12975	141.88				14275	10320	175.61
26-Apr-13	302	20574	9725	181.88	20412	14287	38.93				17132	10803	37.89
3-May-13	309	23935	5808	185.32	24100	14521	30.47				20413	9631	33.22
10-May-13	316	23629	4551	34.93	27670	13380	28.37				16477	9020	20.60
17-May-13	323	16210	6036	26.01	30325	12864	28.76				11103	8732	14.91
26-May-13	332	9981	9778	22.57	17904	10157	17.50				8817	6867	12.86
2-Jun-13	339	9289	7396	16.76	26814	3454	27.14				5763	5069	9.89
14-Jun-13	351	6135	4875	11.18	16284	1600	26.34				3936	2720	10.49
21-Jun-13	358	5590	3089	14.36	11501	7725	27.64				3058	1706	11.92
28-Jun-13	365	4210	1522	12.24	10454	10165	31.03				2336	1477	12.21
8-Jul-13	375	1962	978	13.31	11162	7534	21.93				1839	796	14.87
26-Jul-13	393				4817	4498	10.72						

Supplementary Table S5. Apparent longevity of a honeybee colony in the 2013/2014 experiment at the eclosion rate of 0.9.

This table shows the data source of the apparent longevity in Figure S9, which were estimated using the field experimental results conducted in 2013 to 2014. *a(t)* and *b(t)* denote the number of adult bees and the number of capped brood obtained from the field experiment conducted from 2012 to 2013, respectively (T. Yamada et al., 2018c). *L(t)* is the apparent longevity estimated using *a(t)* and *b(t)* by the mathematical model previously proposed (*1*). "CR-1" and "CR-2" denotes the mite-free control colonies where a pesticide is not administered. "DF 0.2ppm/SS" denotes the mite-free colony where the neonicotinoid dinotefuran of 0.2 ppm is administered via sugar syrup. "CN 0.0.08ppm/SS" denotes the mite-free colony where the neonicotinoid clothianidin of 0.08 ppm is administered via sugar syrup. "FT 1ppm/SS" denotes the mite-free colony where the organophosphate fenitrothion of 1 ppm is administered via sugar syrup. "MT 1ppm/SS" denotes the mite-free colony where the organophosphate malathion of 1 ppm is administered via sugar syrup. **Note:** A pesticide-administration period is from the morning of September 5 in 2013 until December 1 (T. Yamada et al., 2018c).

Date	Elapsed days	CR-1			DF 0.2 ppm/SS			FT 1 ppm/SS			MT 1 ppm/SS			CN 0.08 ppm/SS			CR-2		
		a(t)	*b(t)*	*L(t)*	*a(t)*	*b(t)*	*L(t)*	*a(t)*	*b(t)*	*L(t)*	*a(t)*	*b(t)*	*L(t)*	*a(t)*	*b(t)*	*L(t)*	*a(t)*	*b(t)*	*L(t)*
13-Aug-13	0	7579	4167	23.01	5447	4605	14.97	7360	5094	18.28	5500	4616	15.08	5628	5022	14.18	5953	4683	16.09
24-Aug-13	11	7000	3363	21.26	7556	323	20.76	9290	3758	23.08	6803	2414	18.65	6915	2208	17.43	6041	2146	16.32
1-Sep-13	19	7324	1825	23.77	6529	1219	25.07	10402	2671	27.90	8177	1054	26.11	7790	1025	23.95	5565	558	19.22
5-Sep-13	23	7487	2261	25.14	6017	2214	27.74	10296	3232	28.81	7676	1396	26.84	7293	1506	25.17	5549	578	21.56
15-Sep-13	33	6415	5462	27.73	5072	1743	30.73	5795	6040	22.27	5716	4992	29.32	5574	1825	28.67	4027	2665	26.86
21-Sep-13	39	6697	4683	27.08	5154	90	34.44	7431	3015	24.86	5698	3943	29.32	4001	843	28.20	3829	3198	29.13
27-Sep-13	45	5711	2538	13.68	3317	0	33.45	6920	1655	16.68	5617	2456	22.93	3885	208	30.74	3889	2882	29.88
4-Oct-13	52	5048	649	14.39	3145	0	41.41	5124	1456	17.33	4445	984	14.47	1882	160	19.35	3824	2142	16.20
13-Oct-13	61	4210	151	20.18	2029	0	31.82	4478	11	22.14	3459	0	18.90	1762	7	27.41	4322	113	20.63
27-Oct-13	75	3152	314	31.34	1325	0	41.98	3147	0	33.36	2328	0	30.19	1449	0	40.19	3371	0	29.89
15-Nov-13	94	2714	50	48.37	1146	0	60.17	2554	0	51.13	2050	0	47.95	1143	0	56.81	3181	0	48.19
1-Dec-13	110	2189	3	62.34	795	0	74.23	2213	0	66.43	1745	0	62.35	867	0	70.46	2904	0	63.18
5-Jan-14	145	1100	0	92.61	0	0	0.00	1714	0	100.39	916	0	92.97	0	0	0.00	2218	0	95.60
7-Feb-14	178	0	314	3.81				878	0	124.23	0	0	0.00				1799	0	127.15
28-Feb-14	199	0		0.00				0	0	0.00							1594	0	147.03

Supplementary Table S6. Apparent longevity, *L(t)*, of a honeybee colony in the 2018 experiment at the eclosion rate of 0.9.

This table shows the data source of the apparent longevity in Figure S10, which were estimated using the field experimental results conducted in 2018. *a(t)* and *b(t)* denote the number of adult bees and the number of capped brood obtained from the field experiment conducted from 2012 to 2013, respectively (T. Yamada, 2020). *L(t)* is the apparent longevity estimated using *a(t)* and *b(t)* by the mathematical model previously proposed (*1*). "CR-1", "CR-2" and "CR-3" denote the mite-infested control colonies where a pesticide is not administered. "DF-1 0.4ppm/SS", "DF-2 0.4ppm/SS" and "DF-3 0.4ppm/SS" denote the mite-infested colonies where the neonicotinoid dinotefuran of 0.4 ppm is administered via pollen paste. Note: A pesticide-administration period is from the morning of July 1 in 2018 until the colony extinction (T. Yamada, 2020).

Date	Elapsed days	CR-1			CR-2			CR-3			DF-1 0.4ppm/PP			DF-2 0.4ppm/PP			DF-3 0.4ppm/PP		
		a(t)	*b(t)*	*L(t)*	*a(t)*	*b(t)*	*L(t)*	*a(t)*	*b(t)*	*L(t)*	*a(t)*	*b(t)*	*L(t)*	*a(t)*	*b(t)*	*L(t)*	*a(t)*	*b(t)*	*L(t)*
19-Jun-18	0	8613	5341	20.41	8343	3065	34.45	6987	6861	12.89	9010	5891	19.36	9067	3241	35.40	7079	7726	678.30
1-Jul-18	12	10507	11056	24.90	8439	9401	34.84	9830	8621	18.13	10188	9341	21.89	8926	12549	34.85	10071	5374	471.80
16-Jul-18	27	13064	10657	15.13	12402	7447	22.11	12016	8060	18.56	12070	8360	17.64	13726	8755	14.75	10491	8032	705.20
28-Jul-18	39	12439	5688	14.77	11658	7640	18.81	10037	7006	15.71	11239	5443	16.80	11252	5769	15.88	10301	9052	794.80
12-Aug-18	54	6977	3817	15.81	9812	4619	17.50	6965	4078	13.83	8437	2777	18.96	8588	4030	18.13	9213	4840	425.00
28-Aug-18	70	6284	4125	20.01	9902	6629	22.86	6583	5277	19.15	7609	466	28.70	7558	5690	21.16	5662	4504	395.50
11-Sep-18	84	7369	4755	22.65	8589	5050	16.87	8081	6262	19.30	4716	395	34.80	8742	5928	19.92	4204	2978	261.50
23-Sep-18	96	6427	4201	17.58	9102	5636	20.71	6891	6487	13.93	2370	200	36.12	8046	6559	17.31	1158	1266	111.20
6-Oct-18	109	3680	1490	11.73	8593	5982	19.96	6150	5015	12.22	964	105	35.94	7599	6091	14.92	41	1228	107.80
23-Oct-18	126	1187	658	12.87	6395	4200	15.02	3461	2773	10.97	309	70	28.98	4366	3113	11.52	0	1234	108.30
4-Nov-18	138	596	440	11.46	3373	1747	10.16	1332	1043	6.08	204	43	30.13	1957	1646	7.96	0	0	0.10
18-Nov-18	152	207	411	6.09	383	913	3.73	254	607	3.92	112	39	25.76	191	1147	2.07	0	0	0.00
2-Dec-18	166	56	460	1.54	0	989	0.00	0	691	0.00	0	72	0.00	0	1291	0.00	0	0	0.00
16-Dec-18	180	0	463	0.00	0	0	0.00	0	0	0.00	0	0	0.00	0	0	0.00	0	0	0.00

Supplementary Table S7. Number of mite-damaged bees and mite-prevalence in 2018 and 2011/2012 experiments

2018 (T. Yamada, 2020) and 2011/2012 (T. Yamada et al., 2018a) denote the years when the field experiments were conducted, respectively. CR-1, CR-2 and CR-3 denote the control colonies where no pesticide was administered in the 2018 experiment (T. Yamada, 2020), respectively. DF-1, DF-2 and DF-3 denote the colonies where dinotefuran of 0.4 ppm was administered through pollen paste in the 2018 experiment (T. Yamada, 2020), respectively. DF denotes the colony where dinotefuran of 0.565 ppm was administered through pollen paste in 2011/2012 experiment (T. Yamada et al., 2018a). Mites and Adults denote the number of mite-damaged bees and the number of adult bees, respectively. Mite-prev. denotes the mite-prevalence which is given by dividing the number of mite-damaged bees (Mites) into the number of adult bees (Adults). Incidentally, the numbers of mite-damaged bees (Mites) and adult bees (Adults) and the mite-prevalence (Mite-prev.) in the 2018 experiment (T. Yamada, 2020) and the number of adult bees (Adults) in the 2011/2012 experiment (T. Yamada et al., 2018a) have already been reported. The number of mite-damaged bees (Mites) and the mite-prevalence (Mite-prev.) in the 2011/2012 experiment were newly measured in this work using the photograph images (T. Yamada et al., 2018a) taken in the observation dates selected at intervals of about one month.

	2018																		2011/2012		
Date of observation	CR-1			CR-2			CR-3			DF-1			DF-2			DF-3			DF		
	Mites	Adults	Mite-prev.	Mites	Adults	Mite-prev.	Mites	Adults	Mite-prev.	Mites	Adults	Mite-prev.	Mites	Adults	Mite-prev.	Mites	Adults	Mite-prev.	Mites	Adults	Mite-prev.
19-Jun	1103	8613	0.1281	1577	8343	0.1890	1237	6987	0.1770	1281	9010	0.1422	1795	9067	0.1980	1940	7079	0.2741			
1-Jul	1190	10507	0.1133	1482	8439	0.1756	1986	9830	0.2020	1739	10188	0.1707	2603	8926	0.2916	2457	10071	0.2440			
9-Jul																			84	2158	0.0389
16-Jul	2263	13064	0.1732	2255	12402	0.1818	2670	12016	0.2222	2380	12070	0.1972	3532	13726	0.2573	3488	10491	0.3325			
28-Jul	3475	12439	0.2794	2401	11658	0.2060	3142	10037	0.3130	3351	11239	0.2982	3269	11252	0.2905	3881	10301	0.3768			
6-Aug																			112	4372	0.0256
12-Aug	1876	6977	0.2689	2620	9812	0.2670	2523	6965	0.3622	2689	8437	0.3187	3519	8588	0.4098	3819	9213	0.4145			
28-Aug	2636	6284	0.4195	3313	9902	0.3346	2442	6583	0.3710	2643	7609	0.3474	4008	7558	0.5303	2378	5662	0.4200			
10-Sep																			90	4620	0.0195
11-Sep	3507	7369	0.4759	4125	8589	0.4803	4438	8081	0.5492	3229	4716	0.6847	5988	8742	0.6850	3072	4204	0.7307			
23-Sep	4230	6427	0.6582	6531	9102	0.7175	5793	6891	0.8407	1985	2370	0.8376	6523	8046	0.8107	1002	1158	0.8653			
6-Oct	2903	3680	0.7889	7227	8593	0.8410	5408	6150	0.8793	815	964	0.8454	6919	7599	0.9105	39	41	0.9512			
7-Oct																			104	4232	0.0246
23-Oct	943	1187	0.7944	5575	6395	0.8718	2654	3461	0.7668	247	309	0.7994	3502	4366	0.8021	1	0	1.0000			
4-Nov	292	596	0.4899	2989	3373	0.8862	1240	1332	0.9309	198	204	0.9706	1766	1957	0.9024	0	0		111	3675	0.0302
18-Nov	118	207	0.5700	363	383	0.9478	245	254	0.9646	100	112	0.8929	172	191	0.9005	0	0				
2-Dec	52	56	0.9286	1	0	1.0000	1	0	1.0000	3	0	1.0000	1	0	1.0000	0	0				
3-Dec																			144	2992	0.0481
16-Dec	0	0	1.0000													0	0				

4 Supplementary References

References with an underscore for the first author (three references) are cited in the supplementary material, and the rest are cited in the text.

Alaux C., Brunet J. L., Dussaubat C., Mondet F., Tchamitchan S., Cousin M., Brillard J., Baldy A., Belzunces, L. P. & Le Conte, Y. (2010). Interactions between Nosema microspores and a neonicotinoid weaken honeybees (*Apis mellifera*). *Environmental* Microbiology, *12*(3), 774–782. https://doi.org/10.1111/j.1462-2920.2009.02123.x.

Aljedani, D. M. (2017). Detection of toxicity and effects of some insecticides to local honey bee race (*Apis mellifera jemenatica*). *Journal of the American Chemical Society*, *13*(3), 19–31. https://doi.org/10.7537/marsjas130317.04.

Alqami, A. S. (2006). Influence of Some Protein Diets on the Longevity and Some Physiological Conditions of Honeybee *Apis mellifera L.* Workers. *Journal of Biological* Sciences, *6*(4), 734–737. https://doi.org/10.3923/jbs.2006.734.737.

Amdam, G. V., Rueppell, O., Fondrk, M. K., Page, R. E., & Nelson, C. M. (2009). The nurse's load: Early-life exposure to brood-rearing affects behavior and lifespan in honey bees (*Apis mellifera*). *Experimental* Gerontology, *44*(6−7), 467–471. [PubMed: 19264121]. https://doi.org/10.1016/j.exger.2009.02.013.

Anderson, N. L., & Harmon-Threatt, A. N. (2019). Chronic contact with realistic soil concentrations of imidacloprid affects the mass, immature development speed, and adult longevity of solitary bees. *Scientific. Reports*, *9*, 3724. https://doi.org/10.1038/s41598-019-40031-9.

Aufauvre, J., Biron, D. G., Vidau, C., Fontbonne, R., Roudel, M., Diogon, M., Viguès, B., Belzunces, L. P., Delbac, F., & Blot, N. (2012). Parasite-insecticide interactions: a case study of Nosema ceranae and fipronil synergy on honeybee. *Scientific Reports*, *2*, 326. https://doi.org/10.1038/srep00326.

Aufauvre, J., Misme-Aucouturier, B., Vigues, B., Texier, C., Delbac, F., & Blot, N. (2014). Transcriptome analyses of the honeybee response to *Nosema ceranae* and insecticides. *PLoS ONE*, *9*(3), e91686. https://doi.org/10.1371/journal.pone.0091686.

Barlow V.M., & Fell, R.D. (2006). Sampling Methods for *Varroa* Mites on the Domesticated Honeybee. *Virginia Cooperative Extension* (*VCE*). 444–103. https://vtechworks.lib.vt.edu/handle/10919/50392.

Brandt, A., Grikscheit, K., Siede, R., Grosse, R., Meixner, M. D., & Büchler, R. (2017). Immunosuppression in Honeybee Queens by the Neonicotinoids Thiacloprid and Clothianidin. *Scientific Reports, 7*, 4673. https://doi.org/10.1038/s41598-017-04734-1.

Dainat, B., Evans, J. D., Chen, Y. P., Gauthier, L., Neumann, P. (2012). Dead or Alive: Deformed Wing Virus and *Varroa* destructor Reduce the Life Span of Winter Honeybees. *Applied and Environmental Microbiology*, *78*(4), 981–987.

https://doi.org/10.1128/AEM.06537-11.

Flanagan, D., & Matsumoto, Y. (2016). The Ruby Programming Language (Japanese version translated by Takahiro Nagao under a supervisor of Shohei Urabe), issued the 8th printing of the first edition; O'Reilly Japan Publisher (Selling agency: Ohmsha, Tokyo), Tokyo, July 5, 2016. https://books.google.co.jp/books?hl=ja&lr=lang_ja|lang_en&id=jcUbTcr5XWwC&oi=fnd&pg=PR5&dq=Ruby+Programming+Language&ots=fLBht9bucG&sig=fJuPX7bnJv5MoBDzwAtH2JLpV-A#v=onepage&q=Ruby%20Programming%20Language&f=false.

Forfert, N., Troxler, A., Retschnig, G., Gauthier, L., Straub, L., Moritz, R. F. A., Neumann, P., Williams, G. R. (2017). Neonicotinoid pesticides can reduce honeybee colony genetic diversity. *PLoS ONE*, *12*(10), e0186109 https://doi.org/10.1371/journal.pone.0186109.

Fukuda, H., & Sekiguchi, K. (1966). Seasonal change of the honeybee worker longevity in Sapporo, north Japan, with notes on some factors affecting the life-span. *Japanese Journal of Ecology, 16*(5), 206–212. https://doi.org/10.18960/seitai.16.5_206.

Gregorc, A., Silva-Zacarin, E. C. M., Carvalho, S. M., Kramberger, D., Teixeira, E. W., Malaspina, O. (2016). Effects of Nosema ceranae and thiametoxam in *Apis mellifera*: A comparative study in Africanized and Carniolan honey bees. *Chemosphere*, *147*, 328–336. https://doi.org/10.1016/j.chemosphere.2015.12.030.

Khongphinitbunjong, K., Neumann, P., Chantawannakul, P., Williams, G. R. (2016). The ectoparasitic mite *Tropilaelaps mercedesae* reduces western honey bee, *Apis mellifera*, longevity and emergence weight, and promotes Deformed wing virus infections. *The Journal of Invertebrate Pathology*, *137*, 38–42. https://doi.org/10.1016/j.jip.2016.04.006.

Macedo P.A., Wu J., & Ellis, M.D. (2002). Using inert dusts to detect and assess varroa infestations in honey bee colonies. *Journal of Apicultural Research*, *41*(1–2), 3–7. https://doi.org/10.1080/00218839.2002.11101062.

Martin, S. J., Ball, B. V., & Carreck, N. L. (2013). The role of deformed wing virus in the initial collapse of varroa infested honey bee colonies in the UK. *Journal of Apicultural Research, 52*(5), 251–258. https://doi.org/10.3896/IBRA.1.52.5.12.

Nagata, I., & Yamada, T. (1973). On the parameter-seeking method of local composition equations. *Journal of Chemical Engineering of Japan, 6*(3), 215–219. https://doi.org/10.1252/jcej.6.215.

Omholt, S. W. (1988). Relationships between worker longevity and the intracolonial population dynamics of the honeybee. *The Journal of Theoretical Biology 130*(3,7), 275–284. https://www.sciencedirect.com/science/article/abs/pii/S0022519388800304.

Peng, Y.-C., & Yang, E.-C. (2016). Sublethal Dosage of Imidacloprid Reduces the Microglomerular Density of Honey Bee Mushroom Bodies. *Scientific Reports*, *6*, 19298. https://doi.org/10.1038/srep19298.

Reyes-Quintana, M., Espinosa-Montaño, L. G., Prieto-Merlos, D., Koleoglu, G., Petukhova, T., Correa-Benítez, A., & Guzman-Novo E**.** (2019). Impact of *Varroa destructor* and deformed wing virus on emergence, cellular immunity, wing integrity and survivorship of Africanized honey bees in Mexico. *The Journal of Invertebrate Pathology, 164*, 43–48. https://doi.org/10.1016/j.jip.2019.04.009.

Sammataro, D., Gerson, U., & Needham, G. (2000). Parasitic Mites of Honey Bees: Life History, Implications, and Impact. *Annu. Rev. Entomol.* **45**, 519–548. https://doi.org/10.1146/annurev.ento.45.1.519.

Schmid-Hempel, P., & Wolf, T. (1988). Foraging Effort and Life Span of Workers in a Social Insect. *Journal of Animal Ecology*, *57*(2), 509–521. https://doi.org/10.2307/4921.

Shi, J., Liao, C., Wang, Z., Zeng, Z., & Wu, X. (2019). Effects of sublethal acetamiprid doses on the lifespan and memory-related characteristics of honey bee (*Apis mellifera*) workers. *Apidologie*, *50*, 553–563. https://doi.org/10.1007/s13592-019-00669-w.

Solanki, C., Thapliyal, P., & Tomar, K. (2014). Role of Bisection Method. *International Journal of Computer Applications Technology and Research*, *3*(8), 533–535. https://ijcatr.com/archives/volume3/issue8/ijcatr03081009.pdf.

Strange J.P., & Sheppard W.S., (2001). Optimum Timing of Miticide Applications for Control of *Varroa destructor* (Acari:Varroidae) in *Apis mellifera* (Hymenoptera: Apidae) in Washington State, USA. *Journal of economic entomology*, *94*(6): 1324–1331. https://doi.org/10.1603/0022-0493-94.6.1324.

Straub, L., Williams, G. R., Vidondo, B., Khongphinitbunjong, K., Retschnig, G., Schneeberger, A., Chantawannakul, P., Dietemann, V., Neumann, P. (2019). Neonicotinoids and ectoparasitic mites synergistically impact honeybees. *Scientific Reports*, *9*, 8159. https://doi.org/10.1038/s41598-019-44207-1.

Tackenberg, M. C., Giannoni-Guzmán, M. A., Sanchez-Perez, E., Doll, C. A., Agosto-Rivera, J. L., Broadie, K., D. Moore, & McMahon, D. G. (2020). Neonicotinoids disrupt circadian rhythms and sleep in honey bees. Scientific Reports, 10, 17929. https://doi.org/10.1038/s41598-020-72041-3.

Tarek, H., Hamiduzzaman, M. M., Morfin, N., & Guzman-Novoa, E. (2018). Sub-lethal doses of neonicotinoid and carbamate insecticides reduce the lifespan and alter the expression of immune health and detoxification related genes of honey bees (*Apis mellifera*). *Genitics and Molecular* Research, *17*(2), gmr16039908. https://doi.org/10.4238/gmr16039908.

Vidau C., Diogon M., Aufauvre J., Fontbonne R., Viguès B., Brunet J.-L., Texier C., D. Biron, G., Blot N., Alaoui H. E., Belzunces L. P., & Delbac, F. (2011). Exposure to Sublethal Doses of Fipronil and Thiacloprid Highly Increases Mortality of Honeybees Previously Infected by Nosema ceranae. *PLoS ONE*, *6*(6), e21550. https://doi.org/10.1371/journal.pone.0021550.

Wang, H., Zhang, S.-W., Zeng, Z.-J., Yan, W.-Y. (2014). Nutrition affects longevity and gene expression in honey bee (*Apis mellifera*) workers. *Apidologie*, *45*, 618–625. https://doi.org/10.1007/s13592-014-0276-3.

Wolf, T. J., & Schmid-Hempel, P. (1989). Extra Loads and Foraging Life Span in Honeybee Workers**.** *Journal of Animal Ecology*, *58*(3), 943–954. https://doi.org/10.2307/5134.

Yamada, T., Yamada, K., & Wada, N. (2012). Influence of dinotefuran & clothianidin on a bee colony. *Japanese journal of clinical* ecology, *21*(1),10–23. http://bijensterfte.nl/sites/default/files/Influence%20of%20dinotefuran%20&%20clothianidin%20on%20a%20bee%20colony_Jpn%20J%20clin%20Ecol%20Vol21,%20No1,%2010-23(2012).pdf

Yamada, T., Yamada, K., & Yamada, Y. (2018_a). A clear difference in the impact on honeybee (*Apis mellifera*) colony between the two vehicles of sugar syrup and pollen paste. *Journal of Biological Series, 1*(5), 84–107. https://www.academiapublishing.org/journals/jbs/abstract/2018/Jul/Toshiro%20et%20al.htm.

Yamada, T., Yamada, Y., & Yamada, K., (2018_b). Difference between the impact of the neonicotinoid dinotefuran and organophosphate fenitrothion on a bee colony in a long-term field experiment: an evidence. *Journal of Biological Series, 1*(3), 108–137. https://www.academiapublishing.org/journals/jbs/abstract/2018/Jul/Yamada%20et%20al.htm.

Yamada, T., Yamada, Y., & Yamada, K. (2018_c). 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). *Journal of Biological Series, 1* (4), 187–207. https://www.academiapublishing.org/journals/jbs/abstract/2018/Oct/Yamada%20et%20al.htm.

Yamada, T., Yamada, K., and Apao, P. (2018_d). Comparison of the long-term influence of a pesticide on a bee colony between neonicotinoids (dinotefuran, clothianidin) and organophosphate (fenitrothion) in Maui where there are neither harmful mites nor cold winter. *Journal Biological Series*, *1*(4), 156-186. https://www.academiapublishing.org/journals/jbs/abstract/2018/Oct/Yamada%20et%20al..htm.

Yamada, Y., Yamada, T., & Yamada, K. (2019). A mathematical model to estimate the seasonal change in apparent longevity of bee colony. *Scientific Reports, 9*, 4102. https://doi.org/10.1038/s41598-019-40725-0.

Yamada, T., & Yamada, K. (2020). Comparison of long-term changes in size and longevity of bee colonies in mid-west-Japan and Maui with and without exposure to pesticide, cold winters, and mites. *PeerJ*, *8*, e9505. https://doi.org/10.7717/peerj.9505.

Yamada, T. (2020). Seasonal Changes in the Size and Mite-Prevalence of A Bee Colony Exposed to Dinotefuran via Pollen Paste and Damaged by *Varroa* Mites. *Enliven: Journal of Dietetics Research and Nutrition*, *7*(1), 002. http://www.enlivenarchive-box.org/data/seasonal-changes-in-the-size-and-miteprevalence-of-a-bee-colony-exposed-to-dinotefuran-via-pollen-paste-and-damaged-by-varroa-mites-0.html.

Yang, E.-C., Chang, H.-C., Wu, W.-Y., & Chen, Y.-W. (2012). Impaired Olfactory Associative Behavior of Honeybee Workers Due to Contamination of Imidacloprid in the Larval Stage. *PLoS ONE*, *7*(11), e49472. https://doi.org/10.1371/journal.pone.0049472

Yang, W., Tian, Y., Han, M., Miao, X. (2017). Longevity extension of worker honey bees (*Apis mellifera*) by royal jelly: optimal dose and active ingredient. *PeerJ*, *5*, e3118. https://doi.org/10.7717/peerj.3118.