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Reduction in homing flights in the honey bee *Apis mellifera* after a sublethal dose of neonicotinoid insecticides

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Abstract

The negative effects of a commonly applied systemic insecticide, neonicotinoid, on the honey bee *Apis mellifera* L. are of great concern worldwide, as the use of the chemical is expanding. Recently, special attention has been paid to the sublethal effects of insecticides. An increasing number of studies has identified sublethal effects on the honey bee in the laboratory or in experimental cages, but so far, few studies have examined sublethal effects in the field. To reveal sublethal effects under field conditions, I examined whether the proportion of successful homing flights by foraging honey bees during 30 min after release decreased after bees were topically exposed to insecticides. Honey bees were treated with two types of neonicotinoid insecticide (clothianidin, dinotefuran) and two types of previously common insecticide (etofenprox [pyrethroid] and fenitrothion [organophosphate]) at five different doses (one-half, one-fourth, one-tenth, one-twentieth, and one-fortieth of their median lethal dose - LD_{50}). Then the bees were released 500 m from their hives in the field. The proportions of successful homing flights by bees exposed to neonicotinoids and pyrethroid decreased with doses of one-tenth LD_{50} (2.18 ng/ head for clothianidin, 7.5 ng/ head for dinotefuran) or more and one-fourth LD_{50} (32.5 ng/ head for pyrethroid) or more, respectively, whereas bees exposed to organophosphate did not significantly show a response at any sublethal dose though the trend in decline appeared to. Flight times were not significantly different among treatments at any dose. These results indicate that neonicotinoid and pyrethroid exposure reduced successful homing flights at doses far below the LD_{50} in the field. Moreover, neonicotinoid caused reductions at relatively lower exposure than pyrethroid.

Key words: nonlethal, pollinator, insecticide, clothianidin, dinotefuran, pyrethroid, etofenprox.

Introduction

Pollinators play an important functional role in most terrestrial ecosystems and agricultural fields; they provide a key component of ecosystem services that is vital to the maintenance of plant communities and agricultural productivity. The proportion of animal-pollinated species rises from a mean of 78% in temperate-zone communities to 94% in tropical communities (Ollerton *et al*., 2011). There is empirical evidence of increased crop production in the presence of pollinators in 92 of 108 selected crops (Klein *et al*., 2007). Among pollinators, honey bees, mainly *Apis mellifera* L., are the most economically valued (Moritz *et al*., 2010), and increased yield and quality attributable to bee pollination has been estimated at $14.6 billion in the US crops alone (Morse and Calderone, 2000). It has been estimated that one-third of the food eaten by humans, either directly or indirectly, comes from honey bee pollination (Free, 1993). In addition, the proportions of agricultural crops that depend on honey bees are increasing because of their versatility, low cost, and the ease with which they are moved and managed. The supply of honey bees for pollination, however, has not caught up with its growing demand (Aizen and Harder, 2009). The colony sizes of managed honey bees have been declining over recent decades in Europe and the United States (van Engelsdorp and Meixner, 2010; Potts *et al*., 2010). In particular, mortality during winter has been rising rapidly, leading to widespread losses in the United States (Cox-Foster *et al*., 2007; van Engelsdorp *et al*., 2008; 2009; 2010), Canada (Le Conte *et al*., 2010), Europe (Vejsnaes *et al*., 2010), and Japan (Gutierrez, 2009). Although many factors may account for these declines (Moritz *et al*., 2010; Neuman and Carreck, 2010), one potential factor is the spraying of pesticides on agricultural fields (Oldroyd, 2007; Maini *et al*., 2010; Orantes-Bermejo *et al*., 2010). Initially, research focused on new types of pesticides (i.e., neonicotinoids) because of their translocation properties within the plant (Laurent and Rathahao, 2003). The use of neonicotinoids has rapidly expanded since the launch of the first neonicotinoid compound, imidacloprid, in 1991, accounting for a 16.3% share of insecticide use in crop protection by 2005 (Elbert *et al*., 2008).

To date, many studies have examined the adverse effects of neonicotinoids on honey bees. For example, one previous study found that residue levels of imidacloprid in the nectar and pollen of seed-coated sunflowers under field conditions were below detection (Schmuck *et al*., 2001), imidacloprid residue levels of clover flowers and leaves, wildflowers sampled within imidacloprid-treated potato fields were all below the level of detection (Rogers and Kemp, 2003). In pollen stored within hives sampled from 1,021 apiaries throughout Spain, no neonicotinoid residue was detected, although pesticide residues were found in 42% of samples in the spring and 31% in the autumn (Bernal *et al*., 2010). No residues of imidacloprid were detected in any of the components of the beehives, which were placed at the centre of seed treated sunflower field, and no side effects were observed in the short- or the long-term analysis of the colony growth parameters (Stadler *et al*., 2003). A large-scale survey in the United States in 2007 and 2008 demonstrated that no neonicotinoid residues were observed in bees, although some neonicotinoid compounds

were detected in stored pollen and wax (Mullin *et al*., 2010). Concentrations of neonicotinoid in leaf guttation drops of corn germinated from neonicotinoid-treated seed have previously been reported at levels near those commonly applied for pest control (Girolami *et al*., 2009).

Few studies, however, have demonstrated honey bee decline observed under field is direclty caused by applications by neonicotinoid. For example, Bailey *et al*. (2005) found that pollen collected from neonicotinoid-treated corn and fed to honey bees had no impact on bee mortality, and Nguyen *et al*. (2009) reported that the colony mortality rate was inversely correlated with the surface area of maize fields treated with imidacloprid. A 3-year field survey found no statistical relationship between colony mortality and worker abundance with neonicotinoid residue within apicultural matrices (honey, pollen, and beewax), although imidacloprid was most frequently detected in matrices (Chauzat *et al*., 2009). Schmuck *et al*. (2003) demonstrated that the foraging intensity of bees decreased after spraying with neonicotinoid but recovered within 24-48 h, after which hive vitality was not affected by neonicotinoid. When bee hives were placed in the middle of 1-ha clothianidin seed-treated canola fields for 3 weeks, bee mortality, worker longevity, and brood development did not differ significantly from values in control fields, although clothianidin residues were detected in honey, nectar, and pollen from colonies in clothianidin-treated fields (Cutler and Scott-Dupree, 2007). In summary, most previous studies have reported that neonicotinoid residue has been found within bees and hive matrices, but pesticide levels were far below the dose lethal to honey bees (Maus *et al*., 2003; but see Girolami *et al*., 2009). Thus Decourtye and Devillers (2010) concluded that seed dressed with imidacloprid did not pose any significant risks to honey bee under field conditions, although most scientists agree that further studies are required to elucidate the neonicotinoid effects on honey bee (Ratnieks and Carreck, 2010).

However, studies that have examined the adverse effects of neonicotinoids have been based on recorded mortality levels, with little attention paid to sublethal effects under field conditions (but see Bortolotti *et al*., 2003; Henry *et al*., 2012), which are defined as physiological or behavioural effects on individuals that survive pesticide exposure (Pham-Delegue *et al*., 2002). Ignoring sublethal effects might thus result in an underestimation of the adverse effects of insecticides because the overall impact of neonicotinoids includes sublethal as well as lethal effects (Desneux *et al*., 2007). Among the sublethal effects of neonicotinoids are impairments in learning performance (Decourtye *et al*., 2004a; 2004b; 2005), declines in foraging activity (Medrzycki *et al*., 2003; Colin *et al*., 2004; Decourtye *et al*., 2004a; Ramirez-Romero *et al*., 2005), disruption of homing flights (Bortolotti *et al*., 2003; Yang *et al*., 2008; Henry *et al*., 2012), rise in mortality during winter (Lu *et al*., 2012).

Additionally, these studies of sublethal effects have only been conducted in the laboratory or under semi-field conditions, and most have used only one insecticide and at only one dose levels (but see Decourtye *et al*., 2005). Thus, little information exists concerning the relationship between sublethal doses and the degree of adverse effects, whether neonicotinoids cause more serious sublethal damage than other insecticides, or if the effects observed in the laboratory have ecological consequences in the field. Henry *et al*. (2012) elaborated that oral exposure of imidacloprid at lower dose than LD_{50} reduced the foraging success under field condition. Although neonicotinoid has been focused because of its systemic (Laurent and Rathahao, 2003), recently contact exposure of neonicotinoid has also been revealed to cause serious damages on honey bee (Marzaro *et al*., 2011; Krupke *et al*., 2012; Girolami *et al*., 2012; Sgolastra *et al*., 2012). However, to my knowledge, sublethal effect by topical exposure has not been examined under field condition.

The purpose of this study was to assess the detrimental effect of direct neonicotinoid exposure on homing flights in the honey bee under field conditions. Among the potential sublethal effects, I chose to initially examine the effects on homing flight, as this activity is governed by individual learning and orientation behaviour, which has been repeatedly demonstrated to be adversely affected by neonicotinoids (Colin *et al*., 2004; Decourtye *et al*., 2004a; 2004b; 2005; Ramirez-Romero *et al*., 2005; Yang *et al*., 2008). For this purpose, I examined whether the proportion of successful homing flights by bees was reduced when they were released 500 m away from their nests after being topically treated with one of four types of insecticide (two neocotinoids, one pyrethroid, and one organophosphate) at one of five sublethal concentrations (one-half, one-fourth, one-tenth, one-twentieth, and one-fortieth of its median lethal dose - LD_{50}).

Methods

Insecticides

Four kinds of insecticide that are commonly used in Japanese agriculture fields were used in this study: clothianidin ([E]-1-[2-chloro-1, 3-thiazol-5-ylmethyl]-3-methyl-2-nitroguanidine), dinotefuran (2-methyl-1-nitro-3-[tetrahydrofuran-3-ylmethyl]guanidine), etofenprox (1-ethoxy-4-[2-methyl-1-([3-(phenoxy)phenyl]methoxy) propan-2-yl]benzene), and fenitrothion [O,O-dimethyl O-[3-methyl-4-nitrophenyl]]. The first two insecticides are neonicotinoids, the third is a pyrethroid, and the last is an organophosphate. The LD_{50} levels for dorsal contact toxicities are as follows (values in parentheses indicate ranges of toxicity values reported in the literature): 21.8 (21.8-44) ng/bee for clothianidin (Schmuck and Keppler, 2003; EPA, 2003; Iwasa *et al*., 2004; European Commission, 2005; Frazier *et al*., 2011), 75 (24-75) ng/bee for dinotefuran (Iawasa *et al*., 2004; EPA, 2004), 130 (130-270) ng/bee for etofenprox (Japan Plant Protection Association, 1986; Glattkowski *et al*., 2008), and 130 ng/bee for fenitrothion (Takeuchi *et al*., 1980). Our LD_{50} values were chosen based on the results of a preliminary experiment (Matsumoto, unpublished). Stock solutions (concentrations of 1,000 ppm) of each chemi-

cal were prepared with acetone from standard analytical products: clothianidin (purity 99.9%; Fluka, St. Louis, MO USA), dinotefuran (purity 99%; Wako, Osaka, Japan), etofenprox (purity 99.5%; Wako), and fenitrothion (purity 95.2%; Fluka). Aliquots of the stock solutions were diluted with acetone at specific concentrations immediately before treatments. I exposed bees to insecticides through topical applications because I wanted to simulate the direct doses from foraging in the field. Bees were treated with insecticides at three dose levels (one-half, one-fourth, one-tenth) because I did not know the frequency distribution of insecticide doses on foraging workers under field conditions. In addition two lower dose levels (one-twentieth, and one-fortieth of its LD_{50}) for two neonicotinoids were added because I want to know the threshold, which prevent the successful homing flight.

Homing flight experiment

Experiments were conducted in the field at the National Institute of Livestock and Grassland Science (36.003779°N, 140.009779°E, 21 m a.s.l.). The study field had a straight path that was approximately 600 m long and 5 m wide with fallow and buckwheat fields on one side, a secondary forest of *Cryptomeria japonica* (D. Don) on the other side, and secondary forests of mixed *Quercus myrsinaefolia* (Blume), *Quercus serrata* (Murray), and *Pinus densiflora* (Siebolt et Zuccarini) on the front and back sides. Four bee hives were set up at approximately 1-m intervals at the end of the path on 17 August. Two additional hives were added on 16 September because attacks by hornets, *Vespa mandarinia* Smith, had made it difficult to sample a sufficient number of foraging workers.

I sampled 100 (one-half, one-fourth, and one-tenth of its LD_{50}; for four treatment and control) or 60 (one-twentieth, and one-fortieth of its LD_{50}; for two treatment and control) foraging honey bees using insect nets in front of the hive entrances from 09:00 to 10:30 am on fair-weather days with no strong winds. Sampling was conducted from 2 October to 20 November, and only one hive was sampled per day. Sampled bees were divided into groups of 20 bees, and each group was subjected to one of the following treatments: control (only acetone exposure), clothianidin, dinotefuran, etofenprox, or fenitrothion. Dose levels were one-half, one-fourth, and one-tenth of the LD_{50} values for clothianidin, dinotefuran, etofenprox, and fenitrothion; for clothianidin and dinotefuran, additional dose levels of one-twentieth and one-fortieth of their LD_{50}s were added. To compare among pesticides, I treated all of the bees at the same dose level on a given day. Each dose level trial was performed six times.

Sampled bees were brought to the laboratory and anesthetized briefly with carbon dioxide. Anesthetized bees were exposed to insecticides via topical applications of 1-µl drops of the treatment solution at the specified concentration. The drops were applied to the dorsal side of the thorax using a micropipette (Pipetman, Gilson, France). Afterward, the bees were marked with five poster colors (white, blue, yellow, pink, and orange; Posca, Mitsubishi, Japan). The colour assigned to each treatment was rotated daily. After treatment, each group of 20 bees (representing a single treatment) was put into a cylindrical cage made of metal mesh (9 cm in diameter and 10 cm in height) and left for more than 3 h in the laboratory at room temperature. Bees were given ample food and drink during the resting period by placing cotton balls soaked with sucrose solution (67%) and water within the cages. The bees were released by placing the cages at locations 500 m from the hives and leaving the tops of the cages open. Bees were released from 14:00 to 15:00 hours. The cages were placed at a distance of 500 m because honey bees forage within 4-5 km of their hives, with a peak at approximately 500 m (Sasaki, 1993). I set the release point in the path so that no obstacles occurred between the release point and the hives. For each mark colour, I recorded the number of returning bees in the 30 min after release and the time of arrival in front of the hive entrance. Thirty minutes is selected as a experimental period first because the number of individuals that showed successful homing reached saturation much sooner than this time period (figure 1), and second because labour, time, and energy to spend the observation should be optimized. Bees were released only once per day. Usually, released bees flew away from the cages as soon as the top was removed, although some bees remained within the cages. Bees that remained in the cages after 30 min were excluded from the analyses.

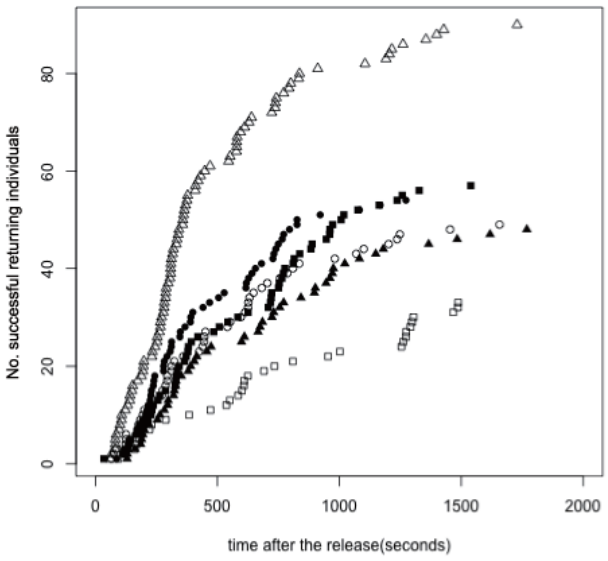

Figure 1. Example of cumulative numbers for successful homing flights by individuals. Relationships between accumulated numbers of successful homing flights by individuals exposed to insecticides by topical application at one-tenth of their mean lethal dose (LD_{50}) values. Four types of insecticide (clothianidin, dinotefuran, etofenprox, and fenitrothion) were applied. Successful homing flights were recorded within 30 min following release at a point 500 m away from the hive. Releases were performed on 3 October (▲), 5 October (●), 7 October (□), 8 October (■), 11 October (Δ), and 14 October (○).

Statistical analysis

I used a generalized linear mixed model (GLMM) (Wolfinger and Oconnell, 1993) with a binomial error structure and a logit-link function to test if the proportion of successful homing flights was reduced by sublethal insecticide doses. The response variable was the homing flights of bees. The explanatory variables were insecticide exposure treatment, period (days) after settling of the hive, and hive identity. I incorporated the period after hive settlement as an explanatory variable because longer settlement periods might increase the proportion of successful homing flights. The analysis of the effect of insecticide exposure was performed in two separate stages. First, likelihood tests were performed using two analytical models. One model included insecticide treatment and period after settling as fixed effects and hive identity as a random effect. Another model included only the period after settling as a fixed effect and hive identity as a random effect. If significant differences between the two models were detected, *post hoc* pairwise comparisons were made between each exposure treatment and the control with Bonferroni corrections. Analyses were conducted in a series of the same dose set because I specifically wanted to know if neonicotinoid exposure caused a more severe reduction in homing flight compared to the other insecticides.

To examine whether insecticide exposure prolonged the flight time to the hive, flight time was analyzed using (GLMMs) with a Gaussian error family and identity as a link function. The response variable was flight time from the release point to the hive. The explanatory variables and analysis procedures were identical to the analysis of the proportion of homing flights. To determine whether the number of bees remaining within cages differed among treatments, the number of bees was also analyzed using GLMMs with Gaussian error family and identity as a link function with treatment and settling time as fixed effects and hive identity as a random effect. All analyses were conducted using R version 2.11.1 (R Development Core Team, 2010).

Results

The proportion of successful homing flights varied significantly among treatments with doses higher than one-tenth of the LD_{50}, but no significant difference was observed with lower doses (figure 2, tables 1, 2). The two

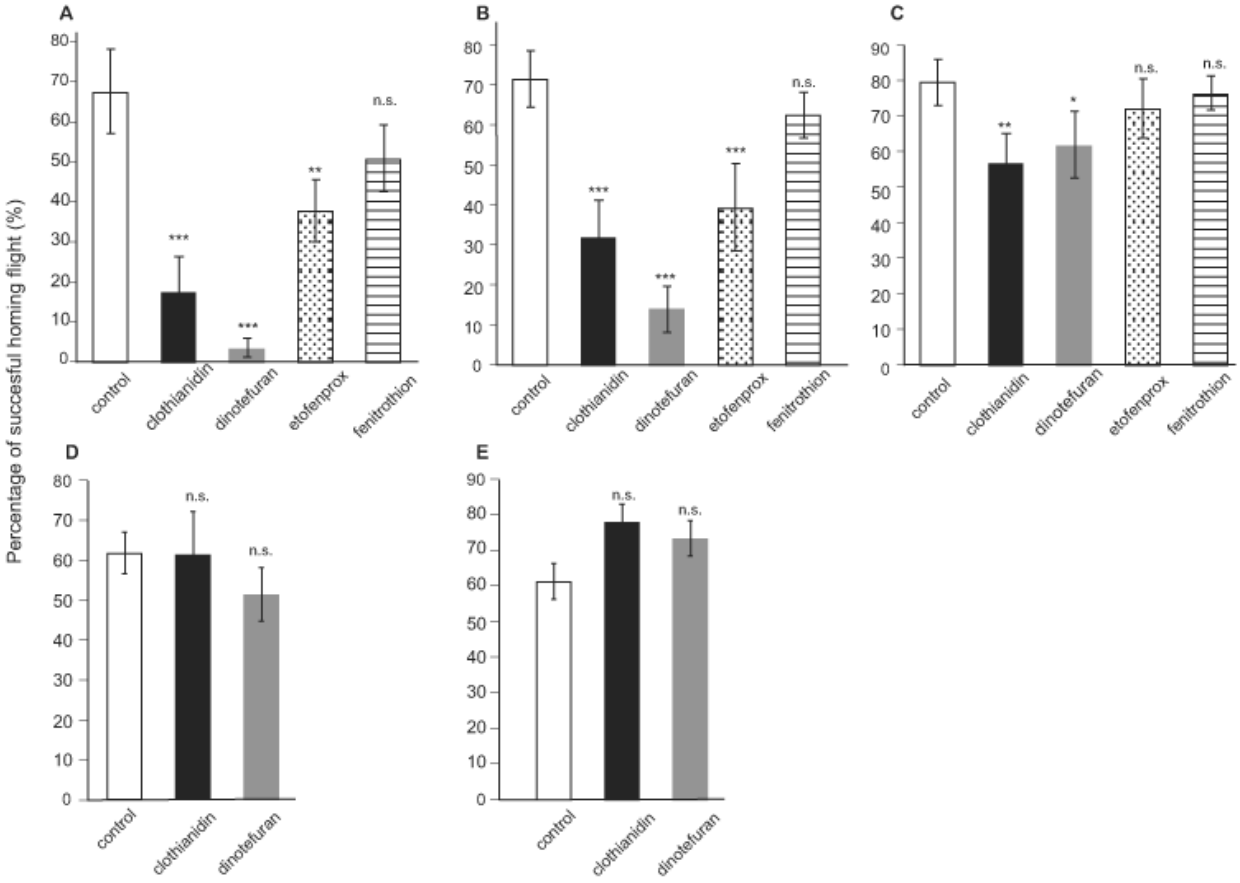

Figure 2. Percentage of successful homing flights in relation to five concentrations of four insecticides. Comparison of the percentage of successful homing flights by individuals exposed to topical application of four types of insecticide (control [white bars], clothianidin [black bars; LD_{50}: 21.8 ng/head], dinotefuran [gray bars; 75 ng/head], etofenprox [dotted bars; 130 ng/head], and fenitrothion [lined bars; 130 ng/head]) at five concentrations (A: one-half, B: one-fourth, C: one-tenth, D: one-twentieth, E: one-fortieth of LD_{50}) within 30 min. Error bars show standard error. Bees were released at a location 500 m away from the hive. Asterisks above bars indicate significant differences from the control after Bonferroni correction (* $p < 0.01$, ** $p < 0.001$, *** $p < 0.0001$).

Table 1. Results of generalized linear mixed models (GLMMs) examining the effects of insecticides on homing flights.

Concentrations fraction of LD_{50}	χ^2	df	P
1/2	110.21	4	<0.0001
1/4	111.26	4	<0.0001
1/10	24.865	4	<0.0001
1/20	1.014	2	0.6023
1/40	3.460	2	0.1773

neonicotinoid treatments (clothianidin and dinotefuran) reduced the proportion of homing flights at doses of one-half, one-fourth, and one-tenth LD_{50} (tables 2a, 2b; figure 2A-C). The etofenprox (pyrethroid) treatment negatively affected the proportion of homing flights at doses of one-half and one-fourth LD_{50} (table 2c; figure 2A-B). The proportion of homing flights with the fenitrothion (organophosphate) treatment was not significantly different from the control at any dose level, although the proportion at one-half LD_{50} was relatively lower than the control (table 2d; figure 2A). No consistent trend was found for the effect on the period after hive settlement (table 3). Significant differences were detected at doses of one-half, one-tenth, and one-twentieth of LD_{50}, although the absolute values of estimated coefficients were extremely small and both negative and positive slopes existed.

For flight time, no significant effect was found except at one-fortieth LD_{50} (table 4). However, after Bonferroni correction, no significant differences were detected between any of the insecticide treatments and the control. The settling period of the hive significantly affected flight time only with a dose of one-tenth of LD_{50} (table 5); the value of the slope was positive. The number of bees remaining within cages did not significantly differ among treatments at any dose level (average 0-13%: $p = 0.16$ for one-half LD_{50}; $p = 0.33$ for one-fourth; $p = 0.84$ for one-tenth; $p = 0.08$ for one-twentieth; $p = 0.12$ for one-fortieth).

Discussion

Among the side effects of pesticides on beneficial arthropods, sublethal effects have recently been gaining more attention (Desneux *et al*., 2007). Sublethal effects occur at levels far below the lethal dose, so damage from pesticides is greater than would be expected without such effects. Therefore, both lethal and sublethal effects should be taken into consideration during risk assessments of pesticides. Although it was suspected that neonicotinoid insecticides have contributed to honey bee losses (Schmuck *et al*., 2003; Oldroyd, 2007), little has been known about whether, how, and to what extent the sublethal effects of the insecticides occur under field conditions (but see Bortolotti *et al*., 2003; Henry *et al*., 2012). This study is the first to show under field conditions that direct topical exposure to two types of neonicotinoid, at doses much lower than their LD_{50}

Table 2a. Results of paired GLMMs with Bonferroni correction examining the effects of clothianidin on homing flight.

Concentrations fraction of LD_{50} (ng/head)	χ^2	df	adjusted P
1/2 (10.9)	54.388	1	<0.0001
1/4 (5.45)	47.739	1	<0.0001
1/10 (2.18)	15.522	1	<0.0001

Paired GLMMs were not conducted for concentrations of 1/20 and 1/40 because the tests did not reveal significant differences between treated insecticides.

Table 2b. Results of paired GLMMs with Bonferroni correction examining the effects of dinotefuran on homing flight.

Concentrations fraction of LD_{50} (ng/head)	χ^2	df	adjusted P
1/2 (37.5)	85.683	1	<0.0001
1/4 (18.75)	2940	1	<0.0001
1/10 (7.5)	13.090	1	<0.01

Paired GLMMs were not conducted for concentrations of 1/20 and 1/40 because the tests did not reveal significant differences between treated insecticides.

Table 2c. Results of paired GLMMs with Bonferroni correction examining the effects of etofenprox on homing flight.

Concentrations fraction of LD_{50} (ng/head)	χ^2	df	adjusted P
1/2 (65)	15.415	1	<0.0001
1/4 (32.5)	30.748	1	<0.0001
1/10 (13)	4.4917	1	0.1362

Experiments for concentrations of 1/20 and 1/40 were not done because the test for concentrations of 1/10 of the chemical did not show significant difference.

Table 2d. Results of GLMMs with Bonferroni correction examining the effects of fenitrothion on homing flight.

Concentrations fraction of LD_{50} (ng/head)	χ^2	df	adjusted P
1/2 (65)	4.342	1	0.1488
1/4 (32.5)	4.485	1	0.1368
1/10 (13)	0.468	1	0.4939*

* *p*-values are non-adjusted because the value with Bonferroni correction exceeded 1. Experiments for concentrations of 1/20 and 1/40 were not done because the test for concentrations of 1/10 of the chemical did not show significant difference.

Table 3. Results of GLMMs examining the effects of settling period on homing flights.

Concentrations fraction of LD_{50}	coefficient ± S.E.
1/2	-0.0209 ± 0.0056 **
1/4	0.0107 ± 0.0101 n.s.
1/10	-0.0239 ± 0.0097 *
1/20	0.0208 ± 0.0053 **
1/40	0.0039 ± 0.0057 n.s.

Estimated coefficients and standard errors (S.E.) are shown for the effects of the period after settling of the hive on homing flights. n.s. not significant, * $p < 0.05$, ** $p < 0.001$.

Table 4. Results of GLMMs examining the effects of insecticides on flight time.

Concentrations fraction of LD_{50}	χ^2	df	P
1/2	4.683	4	0.3214
1/4	6.280	4	0.1792
1/10	7.422	4	0.1152
1/20	0.799	2	0.6750
1/40	6.148	2	0.0462

Table 5. Results of GLMMs examining the effects of settling period on flight time.

Concentrations fraction of LD_{50}	Coefficient ± S.E.
1/2	1.652 ± 2.026 n.s.
1/4	-1.832 ± 1.241 n.s.
1/10	4.787 ± 1.747 *
1/20	0.01191 ± 3.2960 n.s.
1/40	-1.166 ± 4.756 n.s.

Estimated coefficients and standard errors (S.E.) are shown for the effects of the period after settling of the hive on homing flights. n.s. not significant, * $p < 0.05$.

values, caused sublethal effects although Bortolotti *et al*. (2003) and Henry *et al*. (2012) demonstrated that oral exposure of imidacloprid caused the sublethal effect under field condition. According to the fitted dose-response relationship in Cresswell (2011), which was based on a meta-analysis of 14 published studies on the effects of imidacloprid dose through oral intake under laboratory and semi-field conditions, a one-tenth dose (4-8 ng/bee) of LD_{50} of imidacloprid reduced the relative performance of bees by 43-52%. This effect was stronger than my observed reductions in homing flight for clothianidin (29% reduction) and dinotefuran (23%). This difference may be due to the ability of bees to compensate for their degraded ability in field, differences in exposure method (oral versus percutaneous), or differences in insecticide. Regardless, evidence for the sublethal effects of neonicotinoid on bees is accumulating; thus, the effects of neonicotinoids on honey bees must be re-evaluated with consideration of such sublethal effects.

Although growing number of studies have examined sublethal effects, many have been conducted in the laboratory or in cages. Because various factors and their interactions influence the performance of honey bees in the field, the sublethal effects detected on caged honey bees or in a laboratory setting do not necessarily lead to declines in honey bee performance. Thus, the ecological relevance of sublethal effects is still unclear (Thompson and Maus, 2007; but see Henry *et al*., 2012). Further studies under field conditions are needed to fully understand the sublethal effects of neonicotinoids.

In this study, I measured the proportion of homing flights within 30 min of exposure. It is possible that individual honey bees that failed to home could have returned to their hives after my observations were completed. Workers, however, forage with a minimum energy load, which allows a round trip to foraging sites (Sasaki, 2010). Additionally, most of the individuals used in the experiment appeared to return to their hives within 15 min. Thus, it seems appropriate to conclude that almost all foragers that failed to home within 30 min did not return to their hives, even after completion of the experiments.

Settling period was incorporated into models because this factor might accelerate successful homing flight. In fact, settling period at one-half, one-tenth, and one-twentieth of LD_{50} significantly affected homing flight. The coefficients of these effects, however, were very small and the signs were both negative and positive. Because bees actively commute from feeding sites and their hive soon after settling (Matsumoto, personal observation), I suspect that these effects of settling period were pseudo-significant.

Pesticides are almost inevitable in modern intensive agriculture. To promote the use of honey bees in agricultural fields, management methods that prevent adverse effects on honey bees from pesticides, including sublethal effects, are needed. The reduction in homing flights that was caused by the two neonicotinoids was not significant at doses below one-twentieth of their LD_{50}s, suggesting that development of application schemes that keep doses below one-twentieth of their LD_{50}s could potentially prevent possible sublethal effects. To control and limit exposure to levels below one-twentieth of LD_{50}, I need to determine the relationship between insecticide doses in honey bees and the period after spraying, as well as to the spraying volume, pesticide manufacturing methods, and the distance between a hive and a sprayed field.

The sublethal effects identified in this study act on individual foraging workers. Whether the adverse effects on individuals influence the whole colony remains unknown. Whitehorn *et al*. (2012) showed that oral exposure of imidacloprid at the level observed in the field caused reduction in colony weight of bumblebee, *Bombus terrestris* L., which led to the 85% drop in production of new queen. Lu *et al*. (2012) demonstrated that 13-weeks feeding of corn syrup with imidacloprid at the dose level observed at fields to honey bee hives increased mortalities during winter compared to control

hives. Reductions in homing by foraging workers exposed to insecticides may result in reduced foraging activity by the colony, potentially leading to decreased colony growth. Alternatively, an increase in oviposition rate or plasticity in labour division within the colony may compensate for this reduction. To understand the adverse effects of neonicotinoids on honey bee colonies, future research that examines the frequency distribution of neonicotinoid doses on the overall population of foraging workers within a colony, and the consequence of reduced homing flights in terms of colony growth, is required.

Another insecticide, pyrethroid, which was widely used before the recent increase in neonicotinoid use, also reduced the frequency of homing flights like a previous study (Vandame *et al*., 1995). In addition, organophosphate appeared to affect homing flights, but the difference between the treatment and control was only marginally significant. Thus, my results showed that once-common insecticides did reduce homing flights in honey bees, suggesting that the recent rapid loss of honey bee colonies might not be directly linked to the observed decline in homing flight caused by neonicotinoid, whose use expands in agricultural fields. However, neonicotinoids reduced homing flight at lower doses than pyrethroid and organophosphate, and the decline in homing flight caused by contact exposure is only one aspect of potential sublethal effects (Tasei *et al*., 2003).

Because the actual doses of insecticides in the field were not known, by analogy to the hazard quotient (HQ), which in nornally defined by the ration of the application rate to the LD_{50}, I calculate an index of relative risk by direct contact which we define as the concentration of the a.i. in a normal spray application divided by the lowest dose in my experiments at which an effect was detected. Relative risk of fenitrothion is not discussed here because no significant effect was detected for fenitrothion. The clothianidin, dinotefuran, and etofenprox contents within the commercial insecticide are 16%, 10%, and 10%, respectively. Insecticides are commonly diluted 1,000 times for clothianidin, 300 times for dinotefuran and etofenprox with water, and applied with same quantity (25 l per 10 ares; JA Kitahubiki Committee for Rationalization of Fertilization and Control for Pest and Disease, 2011). The two neonicotinoids reduced homing flight at one-tenth of their LD_{50}, (21.8 ng/bee for clothianidin and 75 ng/bee for dinotefuran), and pyrethroid caused reductions at one-fourth LD_{50} (130 ng/bee for etofenprox). Therefore, based on the above relationship, the relative risks by direct contact are 7.339×10^{-5} for clothianidin, 4.444×10^{-5} for dinotefuran, and 1.027×10^{-5} for etofenprox. Thus, the potential relative risk of clothianidin was the highest, at about seven times higher than that of etofenprox. The relative risk discussed here is risks by direct contact, not overall risk of neonicotinoids in agriculture. So far, oral exposure of neonicotinoids through pollen and honey has been focused when it comes to neonicotinoids' effects on honey bee because major mode of application of neonicotinoid is by seed dressing. Recently, Marzaro *et al*. (2011) and Girolami *et al*. (2012) revealed that bees suffered from serious effect thorough direct contact with aerial particles of neonicotinoids during sowing. Thus, I propose that more attention should also be paid on direct exposure. To demonstrate that the higher risk of neonicotinoid contributed to recent declines in honey bee colonies, future research concerning the actual doses of each insecticide affecting bees and consequent effects on honey bee colonies, and the mode posing potential side effects on bees is required.

In conclusion, although neonicotinoid insecticides imposed higher risk on honey bee worker through reduction in homing flight, this study suggested that recent drop in number of honey bee colony is not, at least, primary caused by the neonicotinoid-driven reduction in homing flight observed in this study. Because not only neonicotinoids but also pyrethroid, previous common insecticide, caused the reduction of homing flight, suggesting that pyrethroid driven drop in homing flight probably occurred in field. Of course, sublethal effects by pesticides include not only the reduction in homing flight but also reduction in learning ability, foraging ability, my conclusion does not reject the hypothesis: sublethal effects by neonicotinoid contributes to the honey bee colony drop. I focused on the sublethal effects through direct exposure in the study. Subsequently, we must also explore possible sublethal effects through bee intake of contaminated pollen and nectar.

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