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Imidacloprid and Fipronil induced abnormal behavior and disturbed homing of forager honey bees *Apis mellifera*

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Abstract
Pesticides may be one of causes of recent decline of honey bees (*Apis mellifera*). They may affect the orientation and ability of bees to return to the hive. We investigated the effect of sub-lethal doses of imidacloprid and fipronil on behavior and homing ability of bees. Foragers were injected with different concentrations of insecticides to determine the sub-lethal doses. For imidacloprid, 2, 5 and 10 ng/forager and for fipronil, 1, 5 and 10 ng/forager were found sub-lethal. Foragers caught from hive entrance were injected with sub-lethal doses to observe their homing. Imidacloprid and fipronil induced abnormal behaviors. Treated foragers showed trembling, tumbling, abnormal fanning and grooming, restless running, being stationary, lying on its back and lack of co-ordination. Foragers injected with imidacloprid spent 12.3 minutes on grooming whereas foragers treated with 5 ng fipronil spent 13.8 minutes being stationary. Imidacloprid treated foragers took 54 minutes to recover whereas fipronil treated foragers took 38 minutes. After recovery, foragers were released from 50 m away from the hive to see whether they can come back. Treated foragers showed reduced homing rate. Maximum foragers failed to come back hive. Almost all control foragers were found inside the hive in the next morning of release whereas only few foragers of 10 ng imidacloprid and 5 ng fipronil treated were found. We assume that imidacloprid and fipronil are responsible for bee loss by hampering homing of foragers.

Keywords: Behavior, fipronil, foragers, homing, imidacloprid, sub-lethal dose

1. Introduction
Insect pollination plays a role to benefit the yields of 75% of globally important crop species and 35% of world crop production depends on it [24]. In crop production, the economic value of insect pollination services has been estimated at $153 billion per annum globally [18]. Honey bees are one of the most important insect pollinators because of their role to pollinate natural vegetation and agricultural plants including fruits, vegetables, seed plants, edible oil crops, garden flowers, fiber crops and major forage crops [28]. Worldwide, bees pollinate more than 400 crop species and in the United States more than 130 crop species [22]. Honey bees pollinate 70% of most valuable crops used directly for human consumption [24]. It is estimated that one third of all the plants or plant products eaten by humans are directly or indirectly depends on bee pollination [31]. However, many countries have experienced a loss of insect pollinators specially honey bees in recent years, a situation which threatens ecological stability and global food security [4, 19, 33]. Loss of bees is popularly termed as colony collapse disorder (CCD). This is a phenomenon in which worker bees from a beehive abruptly disappear and only queen and young bees are left in the hive [36]. Beekeepers of Belgium, France, Netherlands, Greece, Italy, Portugal, Spain, Switzerland, Germany and Taiwan have already claimed about CCD [37]. Although CCD is an obvious cause of bee losses, the reasons of the syndrome are not yet fully understood. Till today multiple causes of CCD have been proposed. Some authorities attributed the problem to biotic factors such as parasites and pathogens. Other proposed causes include pesticides, natural habitat degradation, loss of wild flowers, environmental change-related stresses, malnutrition and migratory beekeeping [5, 8, 32]. Many scientists and beekeepers hypothesize pesticides as most crucial cause of CCD. Honeybees are exposed to different agricultural chemicals while foraging on the flowers of blooming crops like sunflower, mustard, maize, blueberry which are regularly treated with pesticides [32]. As a consequence, forager bees contaminate the whole hive if they are able to return to the hive [25]. Due to the concern of the pesticide industry, now-a-days, potential exposure limits for most chemicals are not directly lethal to bees yet sub-lethal doses of certain chemicals can adversely affect bees in

ways which can affect colony fitness [32, 48]. In fact, sub-lethal doses can induce behavioral changes in foraging bees such as memory and learning dysfunctions and alteration of navigational skills [13] and thus foraging activity decreases due to disorientation [7, 11, 23].

Until now, two classes of systemic pesticides- neonicotinoids and phenylpyrazoles, are mainly suspected to cause decline of bees [26]. Among neonicotinoid insecticides imidacloprid and phenylpyrazoles fipronil is used worldwide highly which are directly connected to current bee decline [2, 16]. Imidacloprid is highly potent and selective agonists of nicotinic acetylcholine receptors, which causes nervous stimulation by activating acetylcholine [12, 29, 44] whereas fipronil is antagonist of GABA receptor which hyperpolarizes and disrupts the central nervous system by blocking the passage of chloride ions through the GABA receptor and glutamate-gated chloride (GluCl) channels [39].

Sub-lethal neonicotinoid exposures in honey bees have been reported to induce abnormal foraging activity [42, 49], adversely affect olfactory and visual learning [10, 20, 47], impair the ability to perform the waggle dance [15] as well as impaired orientation skills [3]**.** Moreover, imidacloprid reduced the ability of bees to forage and perform homing flights in field situations [3, 17, 30]. Similarly, fipronil caused disorders of the foraging activity and homing failure of bees [14]. Under semi-- field conditions, bees foraging in sunflower treated with Regent TS® showed abnormal foraging behaviour, increase in grooming, short flights and immobility [6]. Under semi-field and laboratory conditions, bees showed an increase of the foraging period and homing time to the hive, due to a decrease in orientation performance [1]. Fipronil has also shown to act synergistically with pathogens and increased bee mortality [46].

In this study, we tested our hypothesis that sub-lethal doses of imidacloprid and fipronil may cause homing failure in foraging honey bees and thus may be linked to CCD. First, the sub-lethal doses of imidacloprid and fipronil was determined. Second, the behavior of honeybees treated with sub-lethal doses was observed in plastic boxes and finally the homing of those treated bees was observed after releasing them from certain distance.

2. Material and methods

The study was conducted from May to October 2010. Honey bees used in this study were from the colony (*Apis mellifera*) maintained by Prof. Takeshi Ohtani at the Gene Farm of Museum of Nature and Human Activities, Hyogo, Japan. For experimental purpose, in the farm, an observation hive was established inside a room. The observation hive comprised of a comb sheet which is placed in a glass box (60x30x30cm) whose bottom was equipped with heater to maintain an optimum temperature of the hive. One day old honey bees were regularly caught from main hive (cf. photo. 1), marked them on mesosoma with numbered paper disks and put inside the observation hive (cf. photo. 2) for experiment. The observation hive has an isle so that bees can freely exit out and enter in.

Photo 1: Main hives

Photo 2: Observation hive

Imidacloprid and fipronil were purchased from Wako, Japan and Chem Manufacturer, UK respectively. A toxicity test was performed for both insecticides to determine the sub- lethal doses. Imidacloprid was diluted by di methyl sulfoxide (DMSO) and fipronil by ethanol. Foragers (worker carrying pollen or more than 20 days old workers) were caught from observation hive with forceps and individual forager was put in a eppendorf tube. Forager with tube inserted into ice for approximately 45 seconds to anaesthesize the forager. Later, chemicals were injected to foragers through lateral side of mesothorax with a microsyringe. We tested a series of concentration – (2, 5, 10, 15, 20, 25) ng/forager for imidacloprid and (1, 5, 10, 15, 20) ng/forager for fipronil. In this study, 10 foragers were used per treatment in 3 replications for both chemicals. Control group for imidacloprid and fipronil was injected with 0.1% DMSO and 0.1% ethanol respectively. After injection, foragers were put in transparent plastic boxes separately for each treatment. Boxes were provided with sugar syrup as food for foragers. All the boxes then kept into incubator at 25 ± 1 °C for 24 hours. After 24 hours, the number of alive foragers for each treatment were counted.

To test the homing of forager bees, foragers were captured from entrance of observation hive by forceps. Based on the toxicity test experiment for sub-lethal dose determination, foragers were injected with imidacloprid at (2, 5 and 10) ng/forager and fipronil at (1 and 5 ng)/forager. Control group for imidacloprid was injected with 0.1% DMSO and that of fipronil was injected with 0.1% ethanol. Ten foragers were used in 3 replications for each treatment. Anaesthesia and injection method was same as the previous study. Foragers were marked with different colour dot on thorax for each treatment. After injection, foragers were encased in transparent plastic boxes for each treatment and supplied with sugar solution. Behaviors were observed until 30 minutes after injection only for 10 ng imidacloprid and 5 ng fipronil treated

foragers for one replication only. Behaviors of other concentrations treated foragers were not observed because observation by eyes were tedious and difficult. Although foragers showed different behaviors we focused on running (running restlessly here and there in the box), remaining still (stationary on the wall or floor of the box) and grooming (cleaning different parts of body). Three persons observed the behavior and recorded the time spent on each behavior by Ohtani time checker [35]. When all treated foragers showed normal walking and flying tendency inside the plastic cage, they were released from 50 m away of the observation hive to see whether they were able to come back in the hive. Three person took part in this study. One person stayed in releasing site, another one at the outer entrance of observation hive and the rest person observed the hive from room inside. They communicated with each other when any of the marked foragers left the releasing site. Person at the hive entrance ensured that any of the treated foragers went inside and person inside the room record the number of foragers returned. This observation was continued until all foragers left the releasing site. We assumed that some foragers might have gone foraging instead of returning home immediately after release. Hence, observation was continued till evening 17.00 h by which all foragers expected to come back. In case of imidacloprid, treated foragers were searched and counted in observation hive within 1 hour of release and next morning (8.00 h) after release. For fipronil, the numbers were counted within 2, 4 hour of release and next morning (8.00 h) after release.it was observed whether any more foragers returned to the hive upto three days from release by visual searching inside observation hive in the morning 8.00 h.

ANOVA was used to observe the difference among control and treated bees returned home. SPSS 11.5 (SPSS Science, Chicago, USA) was used to analyze the data.

3. Results and discussion

3.1 Determination of sublethal doses of imidacloprid and fipronil on forager honey bees

Despite of many studies regarding lethal doses of imidacloprid [40, 41], there is a great variation mentioned by different authors [43]. Scanty reports are available on lethal and sub-lethal doses of fipronil on honey bees. Hence, preliminary laboratory test was conducted to identify sub-lethal doses for behavior and homing studies. The number of alive foragers was observed after injecting them with a series of concentration of imidacloprid and fipronil over a 24 h period. Foragers injected with DMSO (control), (2, 5 and 10) ng of imidacloprid showed high survival than those treated with (15, 20 and 25) ng of imidacloprid (Fig. 1). At 24 h, only foragers injected with (15, 20 and 25) ng had higher mortality than the control and (2, 5 and 10) ng treated foragers (Fig. 1). For fipronil, foragers treated with (10, 15, 20) ng showed lowest survival rate i.e high mortality than those of control and (1, 5) ng treated foragers (Fig. 2). Several European laboratories found in their laboratory oral 48h LD50= 41-81 ng/bee; contact 48h LD50= 49-102 ng/bee [34]. Other laboratories found even lower values: oral 48 h LD50= 3.7-40.9 ng/bee; contact 48h LD50= 59.7-242.6 ng/bee [41]. Due to causing less mortality, we considered (2, 5 and 10) ng imidacloprid/forager and (1, 5 and 10) ng fipronil/forager as sub-lethal doses for behavior and homing studies.

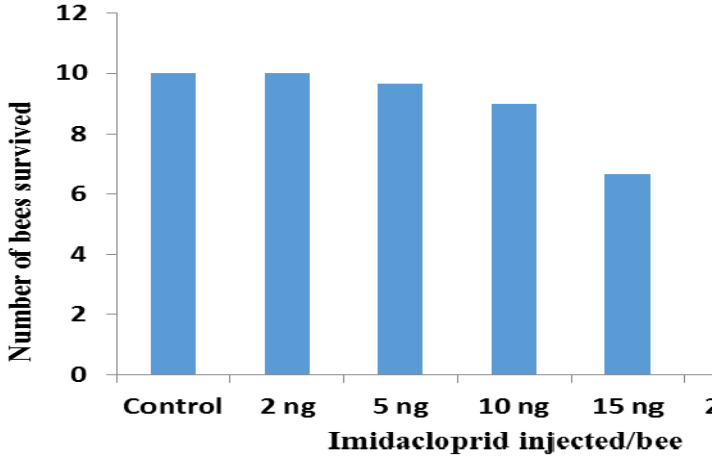

Fig 1: Mean number of bees survived after injecting different concentrations of imidacloprid

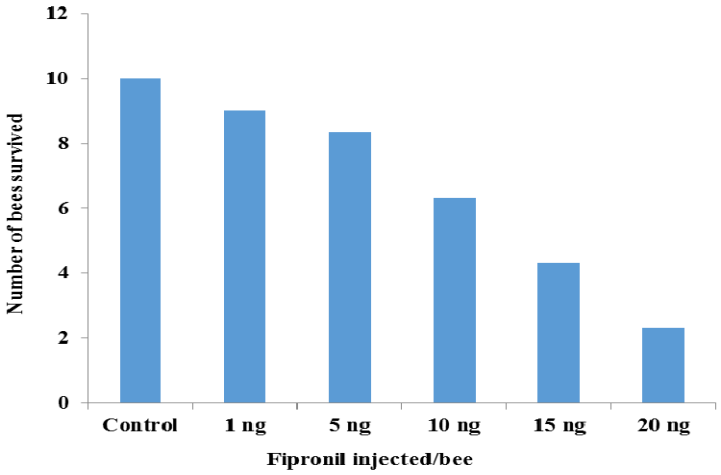

Fig 2: Mean number of bees survived after injecting different concentrations of fipronil

3.2 Effects of imidacloprid and fipronil on behavior of foragers

Sub-lethal doses injection of imidacloprid and fipronil created different abnormalities in forager bees such as trembling, tumbling, vomiting, abnormal fanning, nausea, restless running, being stationary, lying on its back and either remaining still or moving its wings and lack of co-ordination. Our observations on the behavior of foragers are similar to those reported by other authors [43, 46]. In 30 minutes observation, bees treated with imidacloprid spent more time (12.3 minutes) on grooming and less time on restless running (8.8 minutes). They spent 8.9 minutes being stationary (Fig. 3). On the other hand, bees treated with fipronil remained more time (13.8 minutes) stationary followed by 9.3 minutes in grooming and 6.9 minutes in abnormal running (Fig. 4).

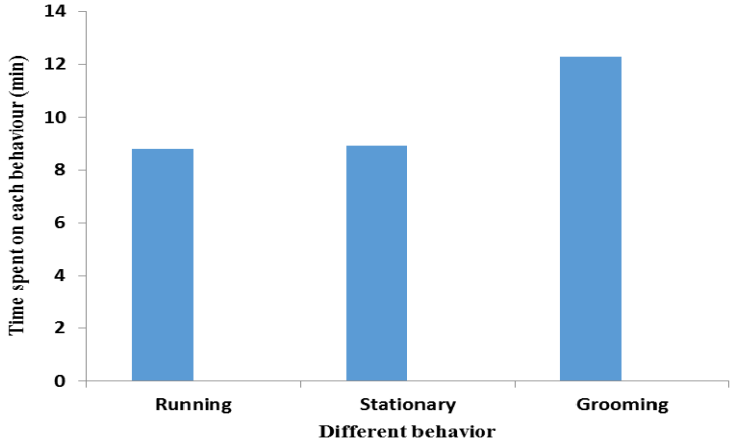

Fig 3: Mean time (minutes) spent by imidacloprid treated bees (10ng/bee)

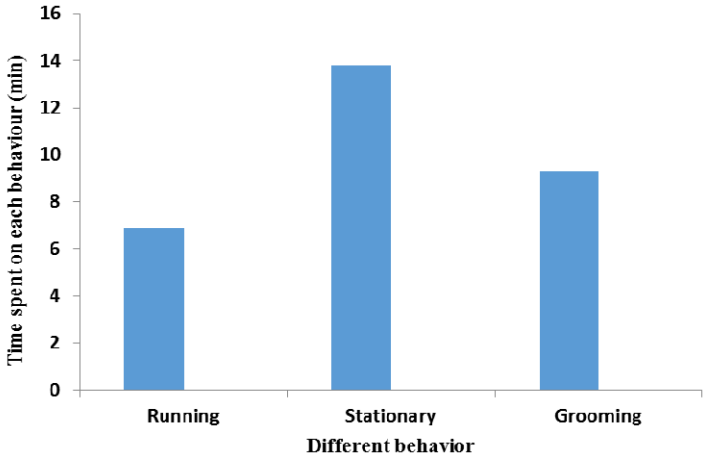

Fig 4: Mean time (minutes) spent by fipronil treated bees (5 ng/bee**)**

Time spent on different behaviors showed variation found from the studies of different authors. In a study by Williamson *et al.* (2014) [46], neonicotinoid treated bees were more likely to lose postural control and spend more time laying on their backs, unable to right themselves. They also reported thiamethoxam treated bees spent more time on grooming. Although we did not observe control foragers the way we observed treated foragers for behavior, we found control foragers spent most of the time in walking. Williamson *et al* (2014) [46] treated their bees by feeding chemical solution and they found 0.45 to 0.54 ng/bee affect bees. Feeding with high doses of imidacloprid, bees spent more time being stationary and less time on walking or running [27]**.** Although we could not observe behavior for all concentrations and for long time, it may be said that imidacloprid and fipronil affect the motor function of foragers such as hampered normal walking, induced abnormal grooming and made them stationary. Imidacloprid showed adverse effects on olfactory learning and memory [10, 47] and impaired the ability of foragers to perform waggle dance [15], suggesting that they perhaps impair motor function. And thus neonicotinoids have subtle behavioral effects on honeybees, which could impair ecologically relevant behaviours such as foraging during pesticide exposure.

3.3 Effect of imidacloprid and fipronil on homing of forager bees

After injecting sub-lethal doses of imidacloprid and fipronil, the recovery time from injection for different concentrations was observed (Table. 1). When bees walked and flied normally inside the plastic box, they considered as recovered. Recovery time was dose-dependent. Bees treated with 10 ng imidacloprid took more time (54 minutes) to recover whereas bees treated with 5 ng fipronil took 38 minutes to recover.

Table 1: Recovery time in minutes after injecting different concentrations of imidacloprid and fipronil

Insecticide	Concentrations (ng/bee)	Recovery time (min)*
Imidacloprid	Control	3 ± 1
	2	4.33 ± 0.57
	5	17.33 ± 2.51
	10	54 ± 5
Fipronil	Control	2.66 ± 1.15
	1	6.33 ± 1.52
	5	38 ± 2.64

*Recovery time expressed as Mean ± SD

Number of foragers that returned to the hive in each treatment during different observation periods are presented in figure 5 and 6. Imidacloprid and fipronil disturbed the homing of forager bees. In case of imidacloprid injection, two observations were made- within one hour after of release and next morning (8.00 h) after release. Because, the foragers were released at 16.00 h and the returned foragers were recorded from 16.00 h -17.00 h. Observation was stopped at 17.00 h as from his long time experience Prof. Ohtani ensured that all foragers come back home within 17.00 h and start go outside after 8.00 h in the morning. Second observation was made in the next morning (8.00 h).

Although the foragers were released when they showed normal walking and flying activities, foragers leaving the plastic boxes influenced by different concentarions of imidacloprid. The control foragers left the box immediately after opening the lid. Foragers treated with 2 ng and 5 ng left the boxes within 15 minutes whereas last bees treated with 10 ng left the box after 35 minutes of opening the lid. Treated foragers also showed abnormal flying behavior. They often fell in the grass and most of their flight direction was not towards the hive. The homing of foragers showed a dose dependent effect. In case of injection of 2 ng/forager, 8.66 ± 1.1 foragers were able to come back home from releasing site within one hour of injection. Injection of 10 ng/forager showed that only 1.0 ± 1.0 foragers came back whereas in control 9.0 foragers came back. Less number of foragers were noticed in all treatments (7.66 ± 0.57, 2.33 ± 0.57, 0.66± 0.57) next morning after release in comparison to what was counted one hour after release. It is speculated that imidacloprid generated abnormal foraging behavior by influencing memories of bees and hence treated foragers left the hive at night. Effect of all the three concentrations injected were significantly different from control according to ANOVA, *p<0.05 (Fig. 10). Restoration of normal activities of returned bees took 2 days. Concentration 10 ng caused complete disappearance of treated foragers (0.66). Missed foragers were seen neither at the hive nor at the entrance or near by places upto 3 days of treatment. During release, treated bees seemed to be disoriented, and that could be the cause of their disappearance.

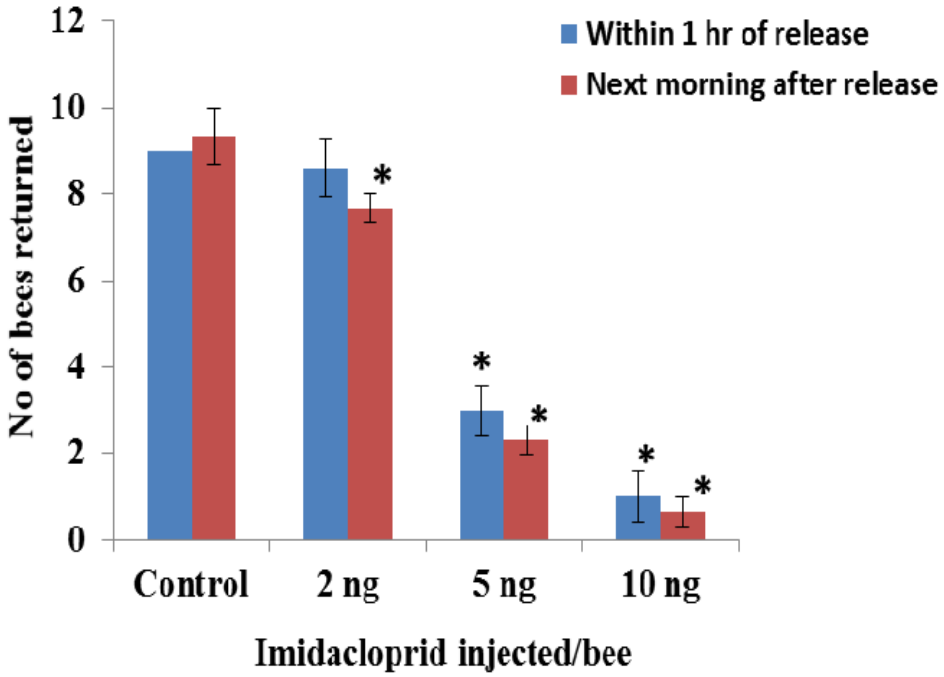

Fig 5: The number of forager bees that return to observation hive after imidacloprid injection. Data is expressed as mean ±SD (n=10). Statistical differences from control were calculated by ANOVA ***p<0.05**

Although 15 ng was sub-lethal, foragers was injected with two concentrations of fipronil- 1 ng and 5 ng due to lack of forager bees in observation hive. The injected foragers was released at 13.00 h and the number of returned foragers was recorded in three observation turns-within 2 hours of release (13.00-15.00 h), 4 hours of release (15.00-17.00) and next morning after release (8.00 h). Like imidacloprid treated bees, fipronil treated foragers also left the boxes at different times after opening the lid. Last forager of 5 ng fipronil treatment left the box after 25 minutes of release. Fipronil treated bees also showed abnormal flying such as resting on grass or tree branches for few minutes while returning home. Some foragers flew opposite direction of their hive, some seated long time in front of entrance before entering the hive. Number of foragers that returned home varied at 2 hr, 4 hr and next morning of release. The number of foragers injected with two concentrations that returned home significantly differed from control. Most of the control bees (8.0±0) returned the hive within 4 hrs of release whereas 5.0 ±1.73 foragers treated with 1ng fipronil and only 2.0±1.0 foragers treated with 5 ng fipronil came back to hive. Injection of 5 ng/bee showed that 1.33±0.33 bees came back within 2 hr of release, 2±1.0 foragers within 4 hr and 2.66±0.57 foragers after next morning of release (Fig.6). Number of returned foragers increased with the time after release suggested that those bees recovered quickly from the effect of fipronil.

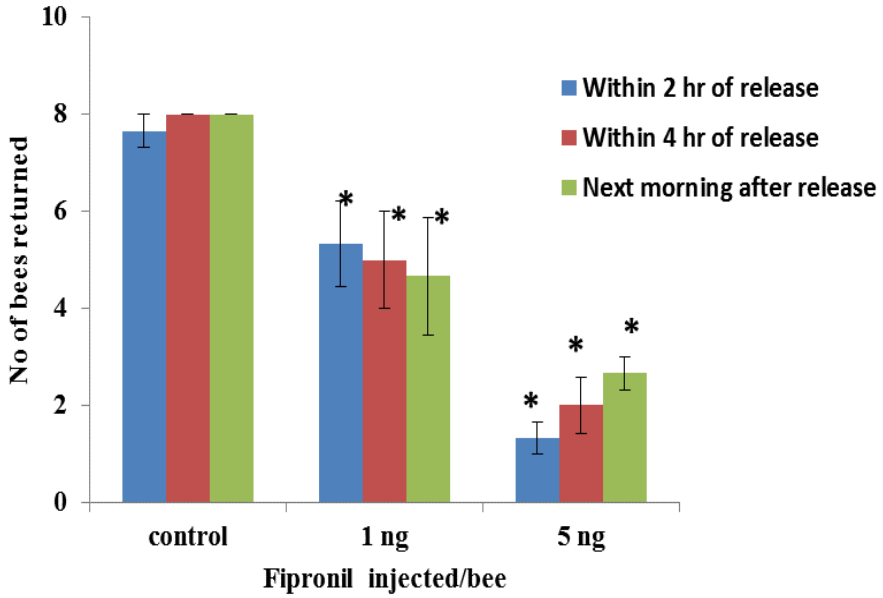

Fig 5: The number of forager bees that return to observation hive after fipronil injection. Data is expressed as mean ±SD (n=10). Statistical differences from control were calculated by ANOVA $^{*}p<0.05$

Both the cases of Imidacloprid and fipronil, the foragers that never came back in the hive were suspected to get lost and died somewhere. Eventually an exposure to such concentrations could therefore strongly affect the hive population. Our results are consistent with those reported by other authors. Bortolotti *et al.* (2003) [3] demonstrated that honey bees fed with 500 ppb and 1000 ppb imidacloprid in sucrose solution fail to return to the hive or to the feeding site. Yang *et al.* (2008) [49] reported that more than 34% of foragers were missing when they treated with imidacloprid with a concentration 600 g/liter and only half of the missing bees turned back to the feeder the next day if they were treated with 6,000 g/liter imidacloprid. Imidacloprid, clothianidin and thiamethoxam have all reduced the ability of bees to forage and perform homing flights in field situations [17, 21, 30, 42]. Curé *et al.* (2001) [7] observed that bees treated with imidacloprid hampers the foraging activities. Bees exposed to sub-lethal doses of imidacloprid, their performance in associative learning and memory tests was impaired and at a certain level of exposure foragers have a higher chance of becoming disorientated and lost [9, 38]. EFSA (2013) [14] reported that fipronil hampered foraging activities and homing failure of bees. Fipronil also increased homing time to the hive, induced abnormal foraging and grooming in forager bees [1, 6].

4. Conclusion

The results lead to the conclusion that both imidacloprid and fipronil disturbed the homing of forager bees. Most of the foragers disappeared after administration of sub-lethal doses of imidacloprid and fipronil probably due to disorientation of the foragers. Treated foragers also seemed to have lost their communicative capacity, which may impair the social behavior within the colony. If the foragers accidentally intoxicated in the field with pesticides, they could feel difficulties in returning to the hive thus reducing the number of foragers in the hive which may lead to the collapse of entire colony. Further studies are needed to investigate the mechanism of disorientation of foragers and to detect where exactly the missing foragers went.

5. Acknowledgement

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