Assessment of Sublethal Effects of Imidacloprid on Honey Bee and Colony Health

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Galen P. Dively and Mike Embrey
Department of Entomology
University of Maryland
College Park, MD 20740

Jeff Pettis
USDA-ARS Bee Research Laboratory
Bldg. 476 BARC-E, Beltsville, MD 20705

Colony collapse disorder (CCD) has caused much concern among beekeepers nationwide. While the specific causes are still unknown, many believe that honey bees have reached a tipping point wherein the colony can no longer protect itself from multiple stresses. One possible stressor is exposure to pesticides used within the hive as well as those used on plants that bees visit for nectar and pollen. Of the potential exposure to pesticides outside the hive, the sublethal effects of the neonicotinoid insecticides on honey bees have been the focus of intensive discussion and research studies in recent years. Many beekeepers and scientists believe that this class of pesticides is a primary stress factor responsible for CCD, although there is no conclusive scientific evidence to directly link neonicotinoids with the disorder.

In this study, we examined the potential sublethal effects of imidacloprid, which is a widely used neonicotinoid applied as a seed treatment, foliar spray, and soil drench on many fruit, vegetable, and field crops pollinated by bees. As a systemic insecticide, imidacloprid accumulates primarily in the vegetative parts of plants and much less in fruiting structures. However, crop residue studies have detected imidacloprid at levels of 2-5 ppb in pollen and >1.5 ppb in nectar of seed-treated corn, sunflowers and rape (review in Maus *et al.* 2003, Bonmatin *et al.* 2004). Recent in-hive surveys of pesticide residues have also found imidacloprid in hive wax and pollen loads (Chauzat *et al.* 2006; Stoner and Eitzer 2008; Frazier *et al.* 2008) but significantly less frequent in samples and at much lower concentrations than other pesticides, especially acaricides used for mite control (i.e. coumaphos, fluvalinate). In a 2007 study funded by the Foundation for the Preservation of Honey Bees, we also found imidacloprid residues at levels as high as 123 ppb in the staminate and pistillate flowers of seedless watermelon treated at planting with the low labeled rate. However, no detectable levels were found in samples of flower rinsate containing pollen and nectar, suggesting that the potential exposure to residues is probably very low if imidacloprid is applied only at planting. Nevertheless, it is conceivable that higher residues of imidacloprid may be present in pollen and nectar of crops if the insecticide is applied closer to bloom.

Although published and unpublished results of sublethal effects on honey bees are conflicting, sublethal exposure to imidacloprid residues may lead to subtle physiological and behavioral abnormalities in honey bees, including decreased queen fecundity, as well as increased susceptibility to other stresses, such as diseases and environmental factors. These effects may be more disruptive to overall colony health than the direct effects on foragers. Most studies to date suggest that imidacloprid can cause disorientation and associative learning problems in honey bees at exposure levels above 20 ppb (review in Maus *et al.* 2003, Decourtye *et al.* 2004). These studies measured sublethal effects on bee larvae or worker bees by exposing

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them to single, small doses of imidacloprid and other pesticides in sugar solution or contaminated pollen. None of the studies examined chronic effects of dietary exposure to imidacloprid in functional colonies over multiple brood cycles. Here, we exposed honey bee colonies for nine weeks to sublethal doses of imidacloprid to examine the potential effects on colony performance and foraging behavior.

Methods and Materials

Packages of 900 g of bees were obtained from a commercial supplier to establish colonies on 4 April, 2008. Sister queens originated from the same breeding line to ensure uniform genetic makeup of bees among treatment groups. We established 30 colonies in nucleus hive boxes containing five foundation frames. All hive equipment was new to eliminate possible carryover contamination. The experimental design consisted of ten replicate colonies assigned to each of three treatment groups: negative control (no imidacloprid exposure); low treatment group exposed to 5 ppb of imidacloprid; and high treatment group exposed to 20 ppb of imidacloprid. The treatments represent the range of concentrations known to be present in pollen and nectar in agricultural crops treated with imidacloprid.

Colonies were located on the University of Maryland research farm at Beltsville, MD in areas free from insecticide exposure. Initially, all hives were managed in one apiary and fed sugar syrup and a pollen substitute (MegaBee patties) to allow colonies to build up before they were assigned to treatment groups (Fig. 1). On 15 May, we inspected colonies to estimate bee and brood densities, and exchanged brood frames among hives to equalize colony strength if necessary. At this time, colonies were randomly assigned to treatment groups and relocated to five isolated apiaries on the research farm. Each apiary consisted of six colonies representing two replicates of each treatment. Individual hives were placed on wood platforms spaced 3 m apart in each apiary (Fig. 2).

Colonies were then allowed to freely forage for nine weeks, during which we provisioned each colony with MegaBee diet either untreated or spiked with imidacloprid. Stock solutions of imidacloprid (Admire Pro, Bayer CropScience) were prepared and diluted in distilled water. We obtained the final concentration for each treatment dose by adding an appropriate concentrated solution of imidacloprid to heavy sucrose syrup, which was then added to the MegaBee powder in a 1.7:1 diet to sucrose solution ratio. This produced soft, moist dough that was formed into 80 g patties. Three or four patties were placed weekly on the top bars of frames inside each colony to allow bees *ad libitum* access to the pollen substitute (Fig. 3). At each diet placement, we removed and weighed remaining portions of the old cakes in order to keep track of the cumulative weight of cakes consumed by each colony. To induce bees to consume a maximum amount of MegaBee diet, we installed pollen traps at the entrance of each hive (Fig. 4).

During the 9-week exposure period, we inspected colonies on a weekly basis to visually estimate the percentage of each frame covered with capped brood, food stores, and bees (Fig. 5). The status of the queen was assessed during each inspection, and whether eggs and successive stages of larvae were present. To prevent overcrowding and the swarming instinct, we removed frames with food stores and brood and added new foundation frames to make room for brood expansion. Queen cells were also removed from combs to prevent swarming. To measure foraging success, we recorded data on the weight of pollen collected each week in the entrance traps, and on the number of foraging bees returning with and without pollen pellets. Foraging counts at the hive entrance were tallied over a 5-minute period in the morning between the hours of 9 a.m. and 11 a.m (Fig. 6). The mixed model procedure of ANOVA (SAS Institute) was used

to test for treatment effects for each measured endpoint of colony performance and foraging. Each hive represented a single experimental unit and apiaries were treated as a random factor. The repeated measures option was used to correct for autocorrelation among weekly samples.

Exposure to the imidacloprid-treated diet ended on 6 August and the pollen traps were removed. We then collected additional data on foraging behavior by marking bees in selected colonies and recording their first appearance and relative numbers at nectar stations at varying distances from the apiary. Each apiary was evaluated one at a time due to limited marking colors and labor requirements. During early evening while bees were returning to hives, an extended entrance chamber was used to collect 300 foragers from each colony. Within each apiary, we selected treatment colonies of similar bee strength, so that any observed differences in foraging behavior would not be confounded by the size of the foraging population. Only one colony from each treatment group was marked in each apiary. Bees from each treatment group were anesthetized with a moderate dose of CO_2 and individually marked on the dorsal thorax with a unique color using an enamel pen (Fig. 7).

Early the following morning, nectar stations consisting of large sponges saturated with diluted honey water were established at various distances (200-500 m) from the apiary. Two to five nectar stations were established around each apiary. We recorded the number of visiting bees marked with each color at each nectar station every 15 minutes (Fig. 8). Counts of marked bees started at 8:30 a.m. and ended when consistent numbers of marked bees from all treatment colonies were observed at each station (usually by 12 noon). Counts of returning marked and unmarked foragers (foraging work force) at each hive entrance were also tallied over a 5-minute period every 30 minutes during the day. We used the number and relative frequency of marked bees visiting nectar stations over time and the number marked bees drifting to other colonies as measures of orientation behavior of foragers and their ability to communicate to other bees. The mixed model procedure of ANOVA tested if foragers from imidacloprid-exposed colonies exhibited the same behavioral tendencies as bees from unexposed colonies.

After the foraging trials were completed on 14 August, 3 g samples of bees and bee bread were collected from each colony and submitted to the USDA-National Science Laboratory at Gastonia, NC, where a multi-residue technique (Lehotay 2006) was used to detect levels of imidacloprid and its two major metabolites down to 0.1 ppb. We also collected samples of 1-week old patties of each treatment for imidacloprid concentration analysis.

Finally, during late August, we transferred the colony and combs of each nucleus hive to a full hive box with an additional five foundation frames added. All hives were then equipped with friction-top pails over the hole of the inner cover and provisioned weekly with sucrose syrup to increase colonies with optimum populations and honey supply for overwintering. On 15 October, we conducted an in depth assessment of colony health and performance by examining each frame to estimate brood development, honey and pollen stores, and queen status. Colonies were then overwintered in the apiaries and observations of survival were recorded the following spring.

Results and Discussion

General observations of colony health. Due to the limited space within nucleus boxes, hive populations became congested, particularly during early June following the spring honey flow. Heavy brood combs were removed and replaced with foundation frames in 26 of the 30 colonies. An average 1.4, 1.6, and 1.4 combs per colony were removed from the control, low,

and high treatment groups, respectively. The congested conditions also triggered 18 colonies to produce queen cells which were removed to prevent swarming. We found no evidence of any clear treatment difference in the number of brood combs or queen cells removed.

Queen failure was relatively high due to the congested hive conditions following the spring honey flow. Nine of the 30 colonies lost queens during early June but were replaced within a week to minimize the effect on brood rearing. Some queen disappearance could have been the result of swarming, although we observed no signs of swarming behavior or noticeable changes in bee strength. Mechanical injury also could have been another reason for the high queen failure, considering the physical manipulations of frames each week. One colony treated with the 20 ppb of imidacloprid became queenless and workers began to lay eggs after four weeks of exposure, making it impossible to replace the drone-laying workers with a new introduced queen. This population eventually declined and died. Another six colonies lost queens during late July and early August but quickly superseded new queens and experienced only a moderate break in brood rearing. Altogether, queen failure occurred in 5 control colonies, 7 low treatment colonies, and only 3 high treatment colonies. These results and the weekly observations of brood rearing revealed no consistent treatment effects on queen health, egg-laying activity, or larval development.

All colonies were relatively free of diseases and pests during the exposure period, except for one control hive on 9 July and one 20 ppb hive on 31 July, when chalkbrood was detected in a few cells. However, later in the summer and early fall, we noted scattered brood cells with chalkbrood and infected mummies on the bottom board in three control, two 5 ppb, and four 20 ppb treatment colonies. Small hive beetle adults and larvae were also detected in six hives (4 controls, one 5 ppb, one 20 ppb) late in the fall but concentrated primarily around untreated MegaBee diet, apparently attracted to the fermenting odor of the patties. All told, the low incidence of chalkbrood and late invasion of small hive beetles did not cause any stress to the colonies, nor were they related to the imidacloprid treatments.

With the exception of the MegaBee patties provisioned to expose bees to treatments, none of the colonies were given supplemental sucrose syrup during June and July after the spring honey flow. Consequently, all colonies depleted their honey supply by the end of August, and fall feeding of colonies after they were transferred to full hive boxes did not adequately build up bee populations and honey stores to ensure winter survival. During early February, we found live bees in the clusters of 14 colonies but none of the 29 treatment colonies survived the winter in the end. Considering the limited space of the nucleus hives and lack of summer feeding, we expected this outcome because the experiment was not intended to test for treatment effects on winter survival.

Diet consumption. During the exposure period, the mean daily consumption of the MegaBee patties ($\pm$SE) was 29.0 $\pm$ 0.84, 29.3 $\pm$ 0.78, and 31.1 $\pm$ 0.85 g in the control, low and high treatment colonies, respectively, and was not different among treatment groups ($F_{(2, 7.2)} = 1.13$, $p = 0.37$) (Fig. 9). Consumption changed significantly over time and peaked on 2 July ($F_{(10, 272)} = 6.11$, $p < 0.001$) but the treatment by time interaction effect was not significant ($F_{(20, 272)} = 1.13$, $p = 0.99$). Studies have showed that MegaBee protein supplement is consumed by bees at about the same rate as pollen-fed counterparts and brood rearing is the same. Though we cannot equate MegaBee consumption directly to natural pollen, it is clear that patty consumption alone represented a significant portion of the daily pollen requirements of a typical nucleus colony (Seeley 1995, Crailsheim *et al.* 1992, J. Pettis, personal communication). Very little bee bread

was stored during and after the 9-week exposure period, which suggests that at least three developmental cycles of brood were exposed to the imidacloprid treatments.

Exposure to imidacloprid was substantiated by residue analysis of bee and bee bread samples collected on 14 August, one week after the final exposure to the treated MegaBee patties. The mean level of imidacloprid ($\pm$SE) was 0.6 ± 0.31, 1.58 ± 0.63, and 3.67 ± 1.48 ppb in worker bees collected from the control, low and high treatment colonies, respectively, and was significantly different among treatment groups ($F_{(2, 22)} = 3.99$, $p = 0.03$). We found similar levels of imidacloprid in bee bread which significantly increased in relation to the exposure concentrations the MegaBee patties ($F_{(2, 20.1)} = 5.80$, $p = 0.01$). The residue level of imidacloprid ($\pm$SE) averaged 0.2 ± 0.22, 1.62 ± 0.68, and 3.49 ± 1.55 ppb in bee bread collected from the control, low and high treatment colonies, respectively. Interestingly, we found traces of imidacloprid in bees and bee bread collected from control colonies and this was probably due to drifting because hives were placed relatively close to each other in the apiaries. We also analyzed week-old patty pieces removed from the hives to confirm the presence of imidacloprid in the MegaBee diet. The mean level of imidacloprid ($\pm$SE) was 0.0, 8.73 ± 1.73, and 15.7 ± 1.45 ppb in patties from the control, low and high treatment colonies, respectively.

Brood development. The percentage of the comb area occupied with capped brood cells was the key indicator of brood rearing. Uncapped (eggs + larvae) brood was also noted during each inspection but not recorded in quantified terms. Recordings of brood rearing started on 28 June after 6 weeks of exposure and continued weekly until 11 September. Overall comb area covered with capped brood was approximately 30% through July and then gradually declined to approximately 20% on the last sampling date (Fig. 10). The time effect ($p = 0.06$) and time by treatment effect ($p = 0.84$) were not significant, indicating that brood development among treatment groups was similar across the sampling period. The overall mean percentage of comb area occupied with capped brood cells ($\pm$SE) was 25.9 ± 1.22, 24.7 ± 1.50, and 29.5 ± 1.36 in control, low and high treatment colonies, respectively, and was not significantly different among treatment groups ($F_{(2, 8.2)} = 1.30$, $p = 0.32$). These results suggest that oviposition rate and larval survival were not impaired, and in fact capped brood was consistently higher in colonies exposed to 20 ppb imidacloprid during the last three sampling dates.

A final assessment of colony performance was conducted on 15 October, which occurred 6 weeks after colonies were transferred to full hive boxes. We found no significant differences in the amount of brood rearing, food stores, and bee strength between imidacloprid-exposed colonies compared to control colonies fed untreated patties (bees, $p = 0.91$; brood, $p = 0.70$; pollen, $p = 0.83$; honey, $p = 0.97$; Fig. 11). The mean ($\pm$SE) percentage of frames covered with bees was 30.5 ± 4.07, 28.0 ± 4.96, and 30.6 ± 4.95, and for capped brood was 7.2 ± 1.36, 6.5 ± 1.04, and 8.0 ± 1.30 in the control, 5 ppb, and 20 ppb treatment groups, respectively. The mean ($\pm$SE) percentage of cells with honey frames covered with bees was 18.4 ± 4.61, 18.2 ± 3.37, and 17.9 ± 3.61, and for pollen was 4.2 ± 0.60, 4.9 ± 1.16, and 4.2 ± 0.80 in the control, 5 ppb, and 20 ppb treatment groups, respectively. The mean percentage of cells drawn on the new foundation frames was very similar in colonies among treatment groups ($p = 0.47$), ranging from 12.8 to 19.2. Brood development declines significantly in all colonies, due to limited amounts of pollen collected during the early fall to stimulate egg-laying. Despite fall feeding with sucrose syrup, levels of stored honey were also less than the amount needed to sustain the overwintering population.

Foraging measurements. Weekly amount of pollen collected in the entrance traps was considered a measure of foraging success. Colonies in the control, low and high treatment groups

collected an average of 19.0 ± 1.33, 21.3 ± 1.70, and 23.6 ± 1.94 g of pollen per day ($\pm$SE), respectively (Fig. 12). We found no treatment effect on pollen weigh ($F_{(2, 9.1)} = 0.21$, $p = 0.81$) or treatment by time interaction effect ($F_{(30, 292)} = 0.98$, $p = 0.50$), but the amount of pollen collected changed significantly over time ($F_{(15, 291)} = 5.02$, $p < 0.001$).

We also measured foraging activity and success by counting the total number of foragers returning to each hive and the percentage of bees loaded with pollen pellets. Weekly counts from 11 June to 14 October varied in response to changing weather conditions and availability of pollen (Fig. 13). Overall mean counts of foragers ($\pm$SE) was 121.6 ± 5.80, 124.6 ± 7.42, and 135.4 ± 7.04 per 5 minutes returning to control, 5 ppb and 20 ppb treatment colonies, respectively. There were no treatment differences ($F_{(2, 13.5)} = 1.03$, $p = 0.39$), nor any indication that foraging activity in the imidacloprid-exposed colonies declined with an increase in exposure time. Likewise, imidacloprid exposure had no effect on the percentage ($\pm$SE) of bees loaded with pollen pellets returning to the control, low and high treatment colonies ($F_{(2, 10.7)} = 1.83$, $p = 0.21$), which averaged 25.9 ± 1.53, 23.2 ± 1.36, and 25.0 ± 1.50, respectively.

In the foraging trials, we recorded three endpoints to measure possible treatment effects on the orientation abilities of bees to find nectar sources and then communicate to other bees. One endpoint was how quickly marked bees from each treatment colony found the nectar sources. Of the 15 nectar stations monitored, marked bees from the control, 5 ppb and 20 ppb colonies were first detected at 9, 6 and 8 nectar stations, respectively. Most first time visits were recorded within 45 to 90 minutes after other unmarked foragers found the source. The second endpoint was the number of returning marked bees recorded at the entrance of the wrong colony. Of the 616 marked bees returning to hives over the five trials, only 5 from control colonies, none from 5 ppb colonies, and 8 from 20 ppb colonies drifted to the wrong hive. In general, data on drifting and first visits to nectar stations revealed little evidence of a treatment effect. The third endpoint was the relative frequencies of marked bees visiting nectar stations in each apiary (Fig. 14). Frequency data are presented as the average percentage of the total number of marked bees recorded at all nectar stations. We analyzed the relative frequencies as a 2-way factorial with treatment colony and apiary as fixed factors and data from individual nectar stations as replicates. A significant interaction effect ($F_{(8, 32)} = 3.04$, $p = 0.01$) indicated that treatment differences that were not the same among apiaries but showed no consistent treatment effect on the number of marked foragers. Of all interaction means, only one pairwise comparison (5 ppb versus 20 ppb treatment means in apiary 5) was significantly different at the 5% probability level.

We also analyzed the frequency data as a one-way ANOVA with apiary as a random factor and individual nectar stations as replicates. Overall average frequency of marked bees from the control, 5 ppb and 20 ppb colonies ($\pm$SE) was 36.0 ± 4.24, 23.3 ± 4.72, and 40.7 ± 4.36, respectively. The treatment effect was significant ($F_{(2, 45)} = 4.11$, $p = 0.02$), indicating fewer visits of marked bees from the 5 ppb colonies but no foraging difference between bees from the control and 20 ppb colonies. The numbers above the mean bars in Fig. 14 indicate the average number of foragers (marked and unmarked) returning per 5 minutes to their respective colonies during each apiary trial. The foraging force differed significantly among colonies in certain trials, even though bee populations looked similar prior to the trials based on inspections of frames covered with bees. Since the number of marked bees represented a very small percentage of the total number of foragers (ranged from 1.8 to 4.7%), there was probably some bias toward stronger colonies in terms of locating nectar stations quicker and then sending out more foragers (including marked ones) to the source. However, the correlation between foraging force and

relative frequencies of marked bees visiting nectar stations was non-significant ($r = 0.04$). Overall, there was no clear indication of any consistent treatment effect on foraging success in finding nectar sources.

Conclusions

Results of this first-year study revealed no treatment differences in the measured endpoints of colony health and no apparent effects of imidacloprid on foraging activities. Some colonies experienced missing queens, signs of swarming behavior, and late summer incidences of disease infections and pests, but these events were not related to the imidacloprid treatments. The lack of sublethal effects after 9-weeks of imidacloprid exposure is encouraging, but further studies are needed before it can be concluded that the imidacloprid residues found in pollen and nectar of treated plants are safe to honey bees.

We will repeat the functional colony experiment in 2009 with the following modifications: 1) use of full hive boxes with half supers to avoid the overcrowding problems and minimize frame manipulation; 2) addition of a positive control (100 ppb) to verify that the bees and brood in the hives are actually exposed and affected by imidacloprid; 3) additional data recorded on bee and pollen pellet weights; 4) changes in the foraging trials to avoid possible confounding effects of foraging force differences; 5) fall feeding to build-up adequate stores of honey to allow for a valid determination of winter survival; and 6) controlled laboratory tests using newly-emerged bees from treatment colonies to examine fitness and colony behavior of imidacloprid-exposed bees and their susceptibility to diseases.

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Figure 1. Nucleus hives with sugar syrup feeders.

Figure 2. Typical arrangement of colonies at one apiary.

Figure 3. Placement of MegaBee patties on top bar of farmes.

Figure 4. Pollen trap attached to hive entrance.

Figure 5. Inspection of individual combs to estimate bee strength, brood, and food stores.

Figure 6. Recording the number of foragers returning to the hive entrance.

Figure 7. Example of marked bees with unique colors.

Figure 8. Monitoring marked foragers visiting a nectar station.
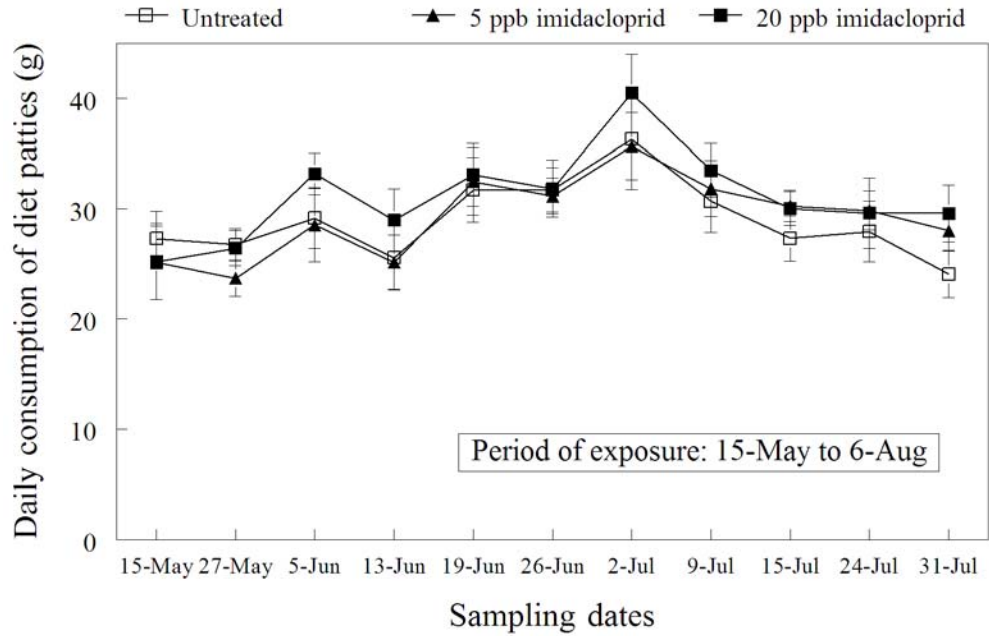
Figure 9. Mean daily consumption (±SE) of diet patties over the exposure period.
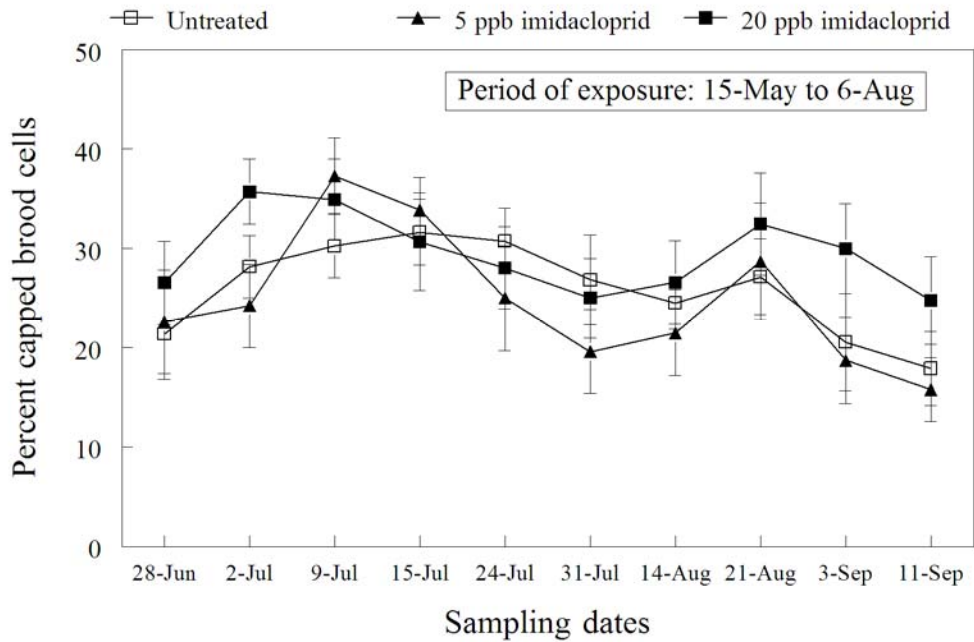
Figure 10. Mean percentage (±SE) of capped brood cells over the exposure period.

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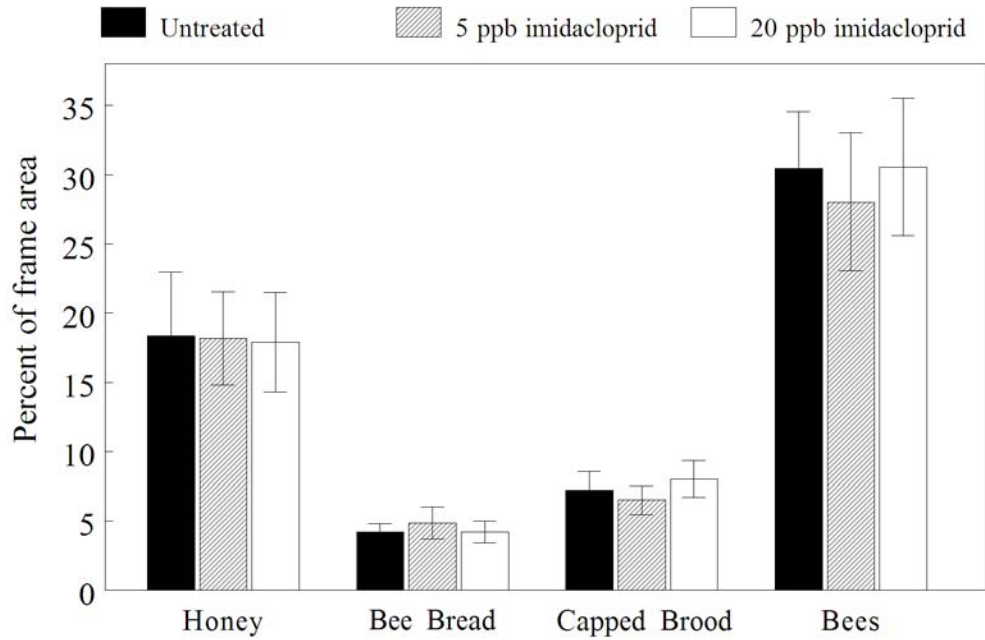
Figure 11. Final visual assessment of colony performance conducted on 15 October. Means (±SE) are given for each colony component.
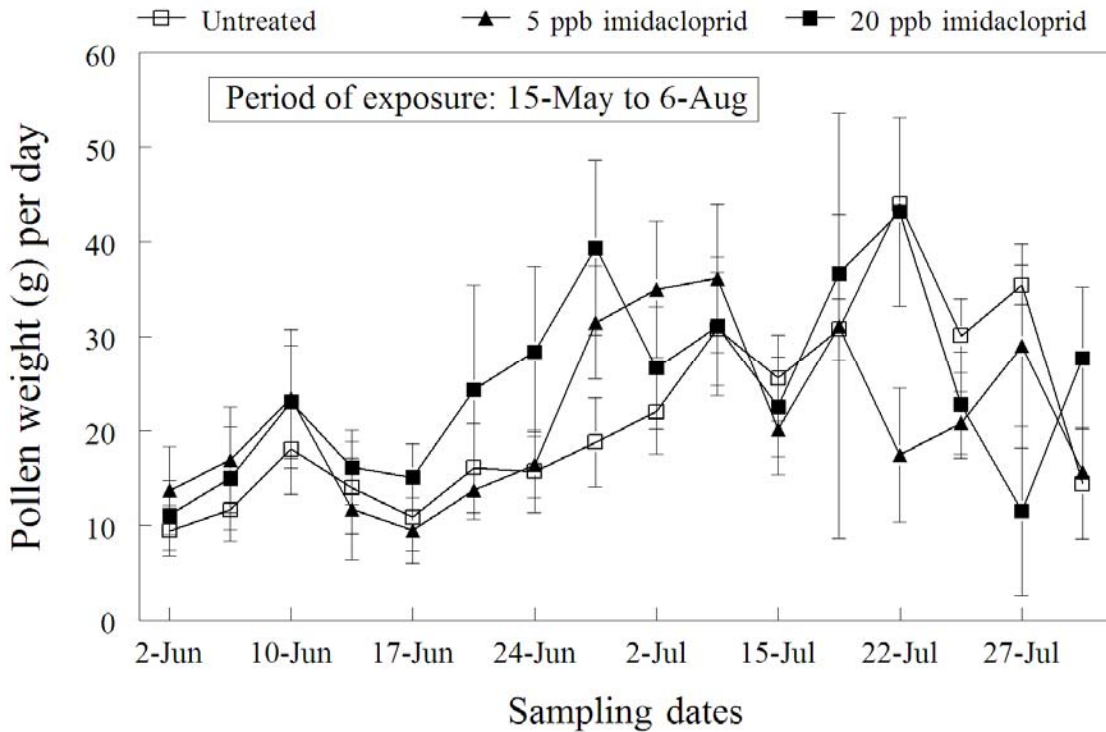

Figure 12. Mean (±SE) weight of pollen collected by the entrance traps over the exposure period.
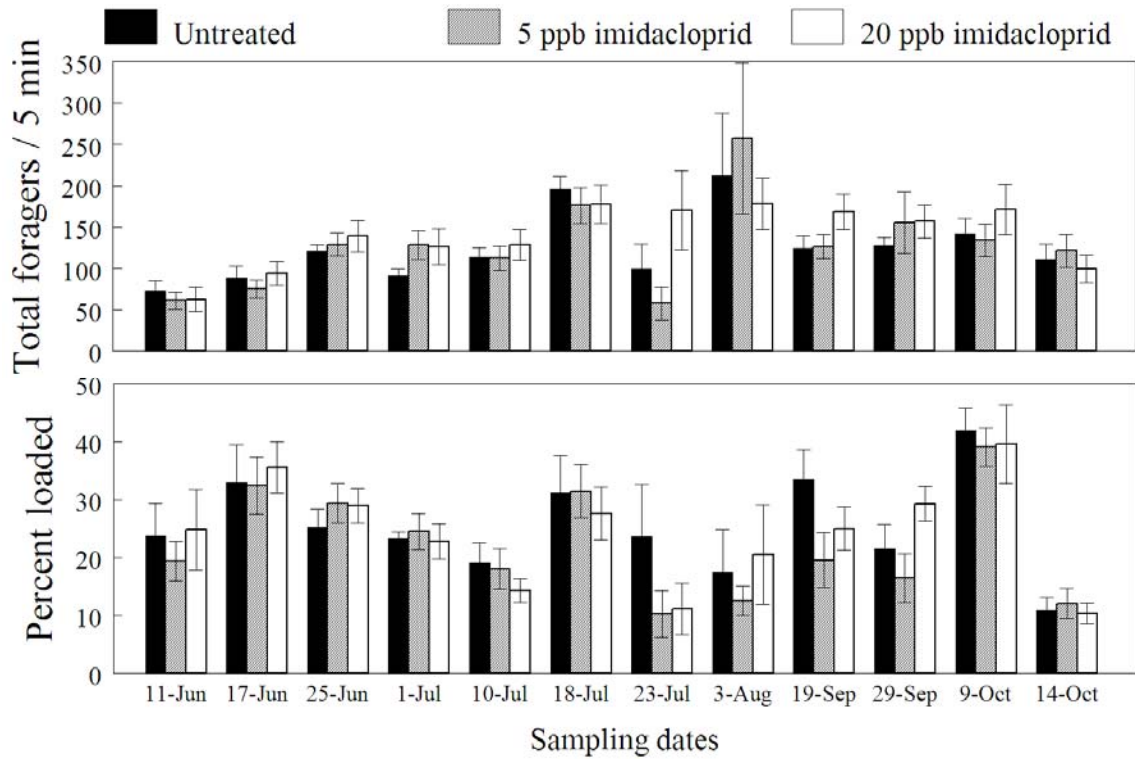
Figure 13. Mean (±SE) number of foragers and percentage loaded with pollen pellets returning to each hive over the exposure period.
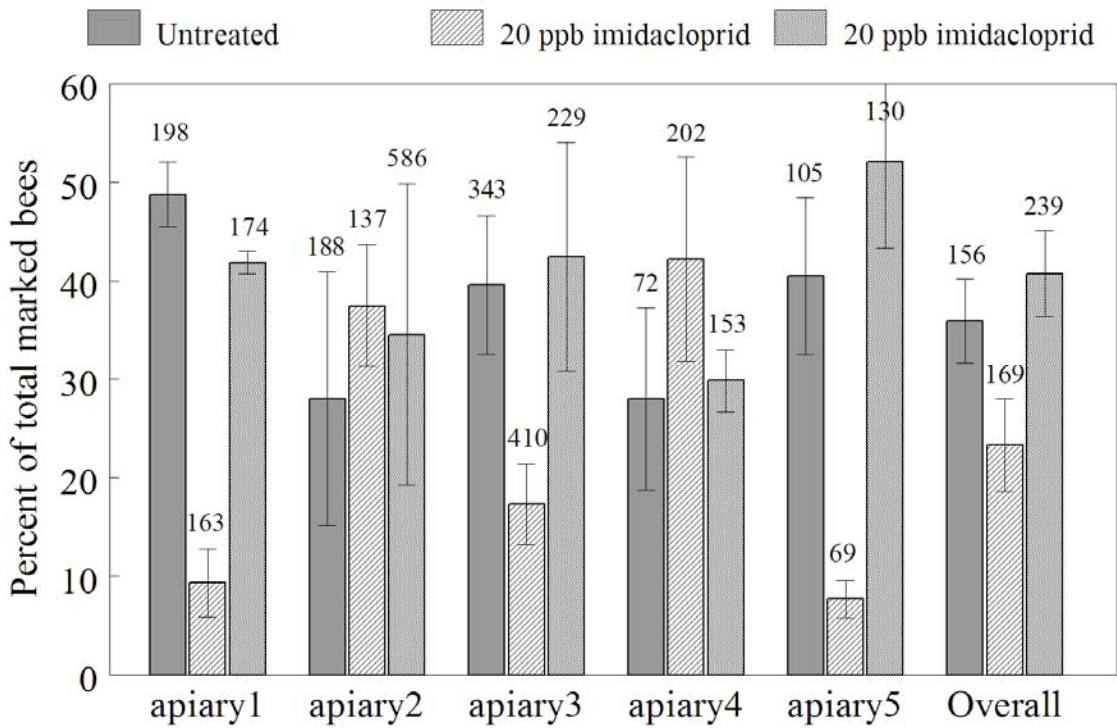

Figure 14. Mean (±SE) frequency of marked foragers visiting nectar stations during each apiary trial. Numbers above bars is the mean number of foragers (marked and unmarked) returning per 5 minutes to their respective colonies during each apiary trial.