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Research article

Apiculture and Social Insects

The Effect of Application Rate of GF-120 (Spinosad) and Malathion on the Mortality of *Apis mellifera* (Hymenoptera: Apidae) Foragers

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Abstract

Beneficial organisms like the honey bee, *Apis mellifera* L. (Hymenoptera: Apidae), are heavily affected by pest control practices that incorporate insecticides. Safer alternatives as the spinosad-based formulation GF-120 have been developed to overcome this issue. Though both the low concentration of spinosad and the ultra-low-volume application rate of GF-120 are supposed to have a low acute toxicity in honey bee foragers, to our knowledge such claims have not been explicitly proven. We thus carried out a series of experiments to assess the effect of GF-120, malathion, and Spintor (spinosad) on honey bee foragers when applied at two concentrations (80 and 1,500 ppm) and two application rates (low density rate [LDR]—80 drops of 5 mm diameter per square meter; high density rate [HDR]—thousands of 200 -μm-diameter droplets per square meter). Interestingly, the three pesticides caused low mortality on foragers when applied at LDR-80, LDR-1,500, or HDR-80. However, HDR-1,500 caused a very high mortality. Based upon these results, we developed a computer program to estimate the average number of foragers that are exposed at LDR and HDR. We found that more foragers receive a lethal dose when exposed at HDR than at the other rates. Our results support the hypothesis that the impact of GF-120 and malathion upon honey bees is minimal when applied at LDR and that computer simulation can help greatly in understanding the effects of pesticides upon nontarget species.

Key words: ecotoxicology, droplet size, exposure, pesticide, simulation

Highly social bees are key species that pollinate plants, both wild and cultivated (Klein et al. 2007); thus, hives of honey bees, bumble bees, and stingless bees are frequently moved into crop areas to increase production (Heard 1999, Velthius and Doorn 2006, Aizen and Harder 2009). During this procedure, it is recommended to minimize the use of insecticides to reduce mortality of bee foragers. Wild bee populations, however, are commonly ignored during pest control actions, though it is acknowledged their positive impact on productivity (Bohart 1972, Garibaldi et al. 2013). Thus, a growing concern regarding the mortality in nontarget organisms caused by pesticides has resulted in the research and development of molecules of biological origin, with a more benign profile than their synthetic counterparts.

Spinosad is a substance constituted of two molecules, spinosyn A and spinosyn D, which are produced by the fermentation of the actinomicete *Saccharopolyspora spinosa* Mertz & Yao (Sparks et al. 2001). It is considered an environmentally friendly pesticide that can be included in integrated pest management strategies and organic farming provided it is used carefully and according to the recommendations of the manufacturer (Williams et al. 2003, Sarfraz et al. 2005). In field assays, spinosad has been found to cause insignificant negative effects to colonies of the honey bee, *Apis mellifera* L.; however, acute toxicity tests in laboratory conditions describe it as a highly toxic compound for individual workers (Miles 2003). The necessity of reducing toxicity of spinosad through increasing its specificity resulted in the development of the formulation GF-120 (NF Naturalyte, Dow Agrosciences, Indianapolis, IN), a special mixture intended for the control of fruit flies. GF-120 owns its environmentally friendly properties to the reduced amount of active ingredient (80 ppm) and the ammonium-based compounds that are attractive to fruit flies but repellent to the honey bee (Mangan and Moreno 2009). Based on laboratory experiments, GF-120 is considered a far less toxic alternative than malathion, the organophosphate widely used to control fruit flies (Peck and McQuate 2000, Mayes et al. 2003). In field tests, Burns et al. (2001) demonstrated that GF-120 does not induce mortality in the short term in exposed honey bee colonies placed in orange groves. Spencer et al.

(2003) obtained similar results in coffee plots, and hypothesized that the rate of application of GF-120 (60–80 drops of 4–6 mm diameter per square meter) could largely explain the low mortality, as the probability that foragers receive sufficient spinosad to kill them is extremely low. In both of these studies, mortality of honey bee foragers and hives was not significantly different to control treatments. Nonetheless, those studies did not consider the ability of honey bees to fly up to 3 km to collect food, so many of the foragers that they observed on the experimental plots could actually come from feral colonies, i.e., those experiments lacked a procedure that assured that the sprayed foragers came unambiguously from the experimental colonies. This situation is highly relevant, as beekeepers from Guatemala and Mexico have claimed that aerial applications of GF-120 cause the weakening, and even loss, of their managed colonies, and the current evidence is mainly based on those studies.

A rapid and low cost approach to gain understanding in many biological processes is the use of computer simulation (Huang et al. 2015). By constructing a computer algorithm, it is possible to create scenarios in which specific hypotheses can be tested. One of the main advantages is that it is not always required to have detailed information about the elements involved in the process to obtain valuable outputs. Thus, it may help to support, or reject, conclusions obtained by experimentation, and vice versa. Moreover, computer simulation allows us to finely tune variables of the experiment that are difficult to control in field or laboratory conditions. Finally, with this tool, we can reduce the number of replicates and of valuable foragers exposed to pesticides needed to test a hypothesis, thus minimizing the stress imposed to our experimental colonies.

In the present study, we investigated if the application rate of GF-120 can actually explain the low mortality in foragers observed in previous works. To achieve this goal, we trained and paint-marked honey bees from a known colony to overcome the issue about the origin of the sprayed foragers. We also developed a computer algorithm that simulates pesticide spraying to gain further understanding on the interaction between pesticide application rate, drop size, and forager density and spatial distribution (cluster or random distribution).

Materials and Methods

Bee Training

Four queenright colonies located in El Colegio de la Frontera Sur, Chiapas, Mexico, were tested in December 2012, December 2013, and October 2014. Honey bee foragers were trained to collect food from a feeder (30-cm diameter, white plastic dish with 50 ml of 50% honey–water solution, placed on the center of a 100 by 100 cm^2, white styrofoam plate), located 20 m to the South of the colonies. When >50 foragers were observed at the feeder, we started the experiments and began to apply the insecticides. To assure that trained workers came from the tested colonies, they were slightly painted on the thorax by using a device developed by Mikery-Pacheco et al. (2013). This device consists of a piece of sponge soaked in paint and held by a piece of fabric mesh; the thorax of the foragers touch the fabric as soon as they exit or enter the colony, getting painted. Any unmarked forager at the feeder was trapped and kept in plastic jars until the end of experiments to prevent recruitment from colonies other than ours, which would make more difficult to handle our marked foragers.

Insecticides and Experimental Design

Initially two insecticides were considered for the study: the spinosad-based toxic bait GF-120 (NF Naturalyte, Dow Agrosciences LLC, Indiannapolis, IN) and the organophosphate malathion (Malathion 1000 CE, SIFATEC S.A. de C.V., Mexico). Both substances were applied at 80 ppm, as this is the concentration of spinosad once GF-120 is diluted and ready for use. However, as it was impossible to obtain a concentration higher than 200 ppm of spinosad using GF-120, we used the concentrate formulation Spintor SC12 (Dow Agrosciences LLC) for the 1,500 ppm treatment. Pesticides were applied to the foragers at the feeder with hand pumps at either 60–80 drops of 4–6 mm diameter per square meter, which is the recommended aerial application rate for GF-120 (low density rate, from now on LDR), or thousands of ~200 micron diameter drops/m^2 (high density rate, from now on HDR). At HDR, ~24,000 drops are sprayed in one square meter at 1 liter per hectare (Matthews 1975). The whole study comprised four experiments, which in turn consisted in three treatments (Table 1).

Insecticide Exposure

Once ~50 foragers were observed on the feeder, water was applied at HDR as a control treatment. Exposed control foragers were collected and caged by placing inverted, cylindrical plastic jars (3-liter volume) on the feeder; the jar was slightly knocked to force foragers inside the jar to move upward, so the cap could be put in place. A small piece of cotton soaked with honey solution was placed inside the jar to feed the foragers. It was normal that some foragers were unloading food in the colony, flying back to the colony, or coming back to the feeder when the application of the pesticides took place, so they were not exposed nor trapped. We offered these foragers a new feeder so they could recruit more nest mates. Again, we waited until ~50 foragers were observed, and a second application with any of the treatments, and according to the experiment, was carried out as shown in Table 1. These foragers were caged as in the control treatment. A new feeder was placed with ~50 ml of honey solution and left until depletion to stimulate recruitment. The next day this feeder was refilled, and the procedure was repeated with a different treatment. Jars were kept in a room at 25°C, 70% relative humidity, and a photoperiod of 12:12 (L:D) h.

Each colony was subjected to each experiment only once; thus, we had four replicates per experiment. We left a 1-wk interval between experiments to avoid pseudoreplication and to allow our colonies to build up their foraging force. Mortality of foragers at 24-h postexposure was determined by visual inspection—any forager that was observed lying on its back, or that did not respond to a gentle touch with a brush was considered dead and removed from the cage. Living foragers were maintained inside the containers and released after the corresponding experiment was concluded. Differences in the 24-h percent mortality between treatments in all experiments were ANOVA analyzed using the core package of R software (R Development Core Team 2012).

Table 1. Application rates, drop size, and concentration of active ingredients used in the four experiments

Pesticide	Experiment 1	Experiment 2	Experiment 3	Experiment 4
Water	HDR-1,500	HDR-1,500	HDR-1,500	HDR-1,500
GF-120	LDR-80	HDR-80	–	–
Malathion	HDR-1,500	LDR-1,500	HDR-80	LDR-80
Spinosad	–	–	HDR-1,500	LDR-1,500

LDR—80 droplets of 5 mm diameter per square meter; HDR—24,000 droplets of 0.2 mm diameter per square meter. The number next to the application rate refers to the concentration of the pesticide, either 80 or 1,500 ppm.

As paint marks could reduce the exposure to insecticides as compared with unmarked foragers, we ran a fifth experiment and applied malathion at HDR-1,500, HDR-80, and LDR-1,500 to unmarked foragers. We ran two replicates of this experiment. These data were compared with the mortality obtained with the respective treatments of the previous experiments using a chi-square test with the core package of R software.

Computer Simulation of Insecticide Exposure

We developed an algorithm in R software (R Development Core Team 2012) that simulates the application of "g" number of drops, either 80 drops of 5 mm diameter (comparable with LDR) or 24,000 drops of 0.2 mm diameter (comparable with HDR), randomly "deposited" over a grid of $10{,}000 \times 10{,}000$ space units (space unit size = 0.1 mm by 0.1 mm), which represents one square meter. We considered the apparent exposure surface area of a honey bee worker as 1.05 cm^2 (Poquet et al. 2014). To simplify calculations, we considered a honey bee to have a circular area of 1 cm^2 while not in flight. An "f" number of bees are previously placed in the grid, either randomly (simulating random foraging) or clustered (simulating recruitment). The algorithm then detects "e" number of bees reached by a drop by simple trigonometric Pythagorean calculation of circle-circle collision. This algorithm was run 100 times with different number of bees, clustered or randomly distributed, at either LDR or HDR.

Results and Discussion

Mortality in unmarked foragers was not significantly different from the mortality observed with marked foragers ($\chi^2 = 5.16$, df = 3, $P = 0.16$); thus, paint marks did not affect the output of our experiments. There was a strong relationship between application rate, pesticide concentration, and mortality. In experiment 1, malathion applied at HDR-1,500 caused 100% mortality, which is significantly higher than GF-120 applied at LDR-80 and water (Fig. 1; $P < 0.001$, $F_{2,\ 9} = 885.7$). In experiment 3, spinosad applied at HDR-1,500 caused a mortality of 100%, significantly higher than the other two treatments (Fig. 1; $P < 0.001$, $F_{2,\ 9} = 438.4$). No significant difference was found among treatments in experiment 2 ($P = 0.08$, $F_{2,\ 9} = 3.45$) and experiment 4 ($P = 0.39$, $F_{2,\ 9} = 1.03$). Thus, both pesticides applied at LDR-80, LDR-1,500, and HDR-80 in any of the experiments did not cause mortality significantly higher than controls.

The simulation agrees with the results from field experiments—though in the HDR simulation a steady increase in the number of bees that are reached by drops is observed in Fig. 2 (randomly placed group), this is only a reflection of the increasing number of bees that are exposed, as on average $90 \pm 0.45\%$ (mean ± SD) bees were reached by drops in all bee densities. This result was not statistically different to $90.7 \pm 0.97\%$ bees reached at HDR when placed in clusters ($t = 0.011$, df = 12, $P = 0.9$; Fig. 2). On average, a maximum of 9 ± 1 drops contacted one bee at HDR. The drop size was set to 0.2 mm diameter for HDR simulations, which corresponds to a volume of ~0.03 µl. Thus, one bee received a maximum of 0.27 µl of product on average. At a concentration of 1,500 ppm, 0.27 µl contains 0.40 µg active ingredient (a.i.), close or above the LD_{50} of malathion (0.40 µg/bee) and spinosad (0.06 µg/bee) for honeybees (Mayes et al. 2003, Hardstone and Scott 2010). That explains why HDR-1,500 caused the highest mortalities in our experiments.

On the other hand, simulations with LDR showed that the actual number of bees that were reached by the drops is very low—$1.60 \pm 0.11\%$ and $1.55 \pm 0.15\%$ for random and clustered bees across

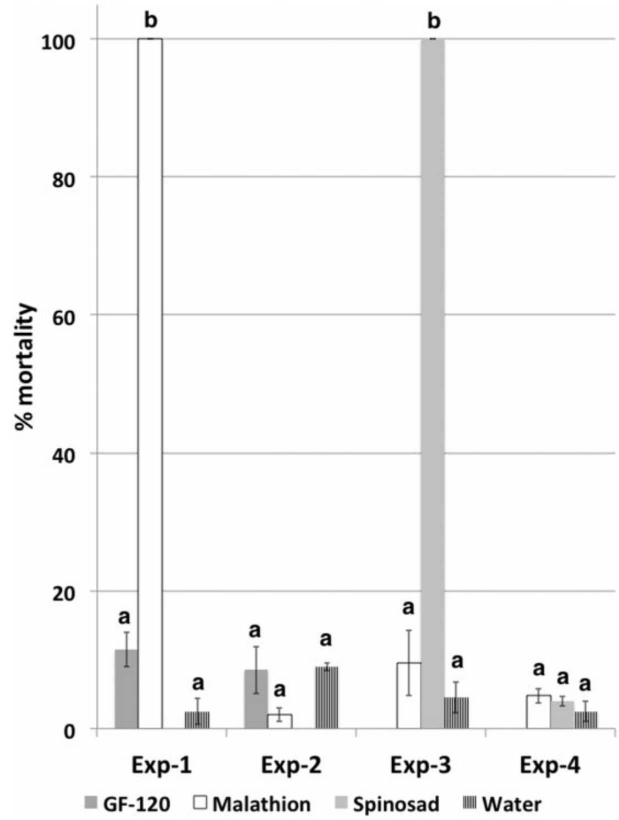

Fig. 1. Mortality at 24 h after exposure to insecticides. Same letters within each experiment indicate no significant difference between treatments (Tukey's honest significant difference test at $\alpha = 0.05$).

all bee densities, which are not significantly different ($t = 0.031$, df = 12, $P = 0.9$; Fig. 2). On average, contacted foragers received one drop, which has a volume of 65 µl. At a concentration of 80 ppm that means 5.2 µg of a.i., much higher than the LD_{50} for malathion and spinosad. At 1,500 ppm foragers would be receiving 97.5 µg, which is far more detrimental. So, although the amount of insecticide per drop is higher in the LDR with large drops, the probability of encounter a drop was much lower, resulting in significantly lower mortality compared with the HDR treatments.

Our experiments and simulations show that the probability that drops of GF-120 reach foragers collecting food during spraying is very low, provided it is carefully applied as recommended by manufacturer. This finding is in agreement with Spencer et al. (2003) hypothesis and the low mortality found in that study. Experiment 2 shows an interesting outcome—if a highly concentrated, 1,500 ppm solution of malathion is sprayed at LDR 80 drops/m^2, the effect upon honey bee foragers will be as low as that caused by GF-120 sprayed at recommended field rate. According to these results, aerial applications of GF-120 as recommended by the manufacturer represent a minor threat to honey bee foragers. It becomes evident that if malathion is aerially sprayed, mixed with a proteinaceous bait, even at 5,000 ppm but at 80 drops/m^2, which is the concentration of malathion recommended for terrestrial spray (SENASICA 2012), the negative effects to honey bees would be significantly reduced and still show activity against fruit flies, provided the susceptibility of wild fruit flies falls below that concentration. This would mean a reduction in both the economic and the ecological burden caused by fruit fly chemical control. For aerial application, 800 ml of GF-120 is used to cover 1 ha, while 200 ml of malathion are occupied. Price

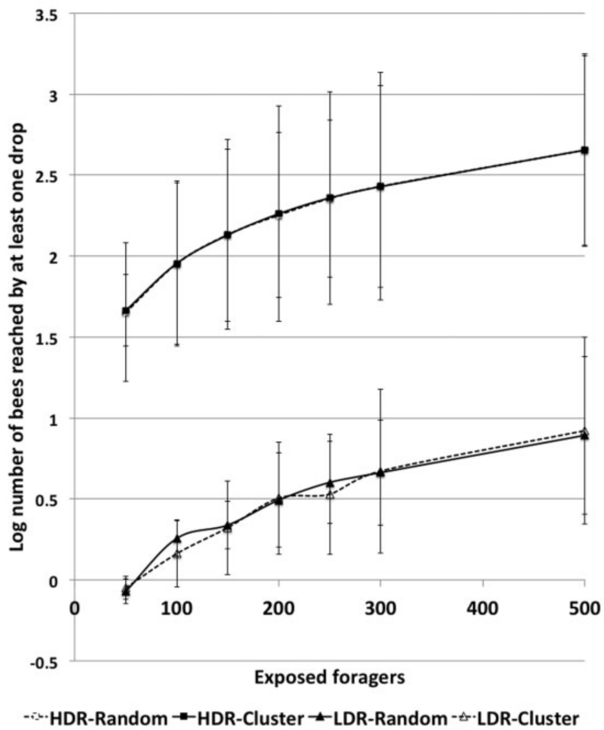

Fig. 2. Number of bees reached by drops in computer simulation. LDR-Random—bees randomly placed on the simulation grid and sprayed at LDR; LDR-Cluster—bees placed on the simulation grid as a cluster and sprayed at LDR. Similar description for HDR-Random and HDR-Cluster curves. Standard error lines are shown. LDR—80 droplets of 5 mm diameter per square meter; HDR—24,000 droplets of 0.2 mm diameter per square meter.

of both substances is roughly US$14.00 per liter in Mexico. It is four times more costly to use GF-120, but it is ecologically friendlier to use it given its biological origin. Moreover, the concentration of malathion applied aerially (190,000 ppm) can be reduced up to 38 times to match the concentration used for terrestrial control (SENASICA 2012). This means that the cost of malathion might be, at least, 152 times lower than that of GF-120, with the corresponding reduction in the effect upon nontarget species. Nonetheless, our simulations suggest that the impact of malathion applied at 190,000 ppm upon flying honey bees would be negligible if applied at 80 drops of 5 mm diameter per square meter; but it also means that more malathion reaches environment and that entering in contact with even minute amount of this solution could kill an insect.

Interestingly, our simulations reveal that the impact of application rate is independent of forager density, and that estimation of the proportion of foragers that are contacted by the drops can be carried out using only two variables: coverage of the pesticide (drops/m^2) and drop size. With our algorithms, it is possible to estimate the number of drops, and thus the amount of pesticide, that reach a bee forager, or any other insect of any size. Having this information in advance, the following step is to validate it with a few field or laboratory experiments. Here is where the power of computer simulation relies. We are currently planning in using this approach to evaluate the impact of many pesticides in other bee species, like stingless bees and solitary bee species, for which nothing has been done in the study region and little biological material is available.

Though GF-120 is considered repellent to honey bees, we did not observe such effect in our experiments: not all foragers were discouraged from feeding when GF-120 was applied. Actually some GF-120 drops fell in the food, but foragers continued collecting it, as observed with the stingless bee *Plebeia moureana* by Sánchez et al. (2012) and Gómez-Escobar et al. (2014) with other bee species. However, we think that GF-120 is still safe, as we did not find a significant difference between control and LDR-80 treatments. However, to further reduce the impact upon pollinators, more effective repellents should be investigated to achieve a complete repellence in honey bees.

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