Learned Associations and Preferential Foraging in Honey Bees (*Apis mellifera*)

Veronica Thomas, Biological Foundations of Behavior, Animal Behavior

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Figure 1. The number of bees visiting each testing station at five-minute increments.

Figure 2. The total number of bee visits to each test condition over an hour period for both test days combined.
Acknowledgments

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Abstract

This study examined whether or not it was possible to train bees to avoid plants treated with a synthetic herbicide. During the training period, I taught a colony of honey bees to associate one odor (mint-lemongrass oil) with a high-reward food (2:1 sugar water solution), and another odor (grapefruit oil) with a low-reward food (water). For the test, 24 organically-certified chrysanthemums were obtained. Eight chrysanthemums were treated with the mint-lemongrass oil, eight more were treated with grapefruit oil, and the final eight were treated with grapefruit oil and a synthetic herbicide. The foraging patterns of the bees were recorded over three one-hour periods. The bees preferentially foraged at flowers treated with mint-lemongrass oil. They also preferred flowers without synthetic herbicides. The results of this study demonstrate that 1) bees can transfer a learned association into new contexts, and 2) bees may naturally prefer to forage on plants that have not been treated with herbicides. This training can be used to help bees more effectively avoid plants treated with potentially harmful chemicals.

Introduction

Honey bees (*Apis mellifera*) are a vital link in many different ecosystems and contribute to the success of one-third of all agricultural practices (vanEngelsdorp 2009). They are also valued as an important source of scientific information on social insects, providing information on how colonies function together. Honey bees display many unique behaviors that not only help in their foraging success, but also maintain a successful hive environment. The social systems of honey bees are very well understood (Bouton 2007), but bees are also a valuable study organism for many other aspects of animal behavior.
Honey bees have long been studied for their learning abilities. In order for honey bee foragers to locate quality food sources, they must be able to both learn the location of a variety of ephemeral food sources and the olfactory and visual cues associated with them. In laboratory studies, honey bees have been shown to associate specific odor cues, color cues, or combined odor and color cues with particular food sources (Bhagavan and Smith 1996). Trained bees have also been shown to return to valuable food sources and to communicate their location to other members of the hive (Hammer and Menzel 1995). In addition to being able to pair specific odor or color cues with a reward, bees can also identify reward gradients (Bouton 2007). They not only can associate an odor with a reliable food source, but they can also associate cues with unreliable food sources, and use those cues to evaluate foraging alternatives (Bouton 2007). In addition, studies have shown that bees can not only learn specific odor and food pairings, but can then learn to reverse these associations, possibly because of the changing, ephemeral nature of their natural food sources (Komischke et al. 2002).

In general, two types of appetitive training procedures are used for bee studies. The first is called free flying conditioning. In this system, bees are captured from a feeding station near the hive and transported to the lab (Bouton 2007). At the lab, they are exposed to a training design, and are then marked and allowed to leave and return to the training station on their own (Bouton 2007). The second type of training is called proboscis extension training (Gil and DeMarco 2005). In this design, bees are harnessed in a metal tube and restrained with tape. A sucrose solution is then touched to their antennae, which triggers extension of the proboscis. This is a reflexive action (Gil and DeMarco 2005). Bees can be trained associatively if an odor or color is presented and immediately followed by the presentation of sucrose solution. Training is then measured by the amount of proboscis extension (Gil and DeMarco 2005). A method similar
to proboscis extension has also been used in aversive conditioning, where presentation of a stimulus is paired with a shock, and stinger extension is used to monitor training effectiveness (Vergoz et al. 2007).

Despite the importance of honey bees across ecosystems and their critical role in many agricultural practices, populations of bees are decreasing rapidly due to a widespread problem referred to as Colony Collapse Disorder, or CCD. Colony Collapse Disorder first appeared in the winter of 2006 to 2007, and has reduced populations of honey bees globally by up to 90% (vanEngelsdorp et al. 2009). In general, it is characterized by the rapid loss of adult worker bees, with a lack of dead bees occurring within or in close proximity to the hive (vanEngelsdorp et al. 2009). Additionally, there is an obvious and somewhat unusual lack of hive robbing from other organisms or bees (vanEngelsdorp 2009). The causes of CCD are still not entirely understood, but are likely due to a variety and combination of different stressors, such as exposure to synthetic chemicals in the form of pesticides and herbicides (Decourtye et al. 2005), diseases such as Kashmir Bee Virus, and parasites like varroa mites (vanEngelsdorp 2009). Early research into CCD has indicated that, while synthetic chemicals alone are probably not the direct cause of Colony Collapse, exposure to sub-lethal doses of pesticides and herbicides may place hives at a higher risk of contracting potentially fatal diseases (Bernal et al. 2010, vanEngelsdorp et al. 2009). In addition, some research indicates that exposure to synthetic chemicals may decrease the foragers’ ability to process olfactory cues. The ability to avoid plants that have been exposed to these harmful chemicals, then, would be highly beneficial to bee populations and the humans that depend on them (Decourtye et al. 2005).

In this experiment, I plan to investigate whether honey bees trained to approach or avoid particular scents associated with variable food sources can transfer this learned association to
real plants, some of which are organic, while others are treated with chemicals. It is important first to see if the bees have an existing preference for either chemically treated plants or organic plants and for either of the scents used in the learned associations. If there is no existing preference, it is then important to see if a preference can be created using trained olfactory cues that signal either high or low reward food options. I predict that the honey bees will be able to associate positive and negative rewards with different odor cues, as was indicated in previous research that used this experimental design (Bouton 2007). I also predict they will successfully transfer this association to the new context, as honey bee learning abilities are fairly similar to other organisms that have demonstrated the ability to transfer positive associations to new contexts (Bitterman 1996).

Methods

The experiments were carried out within 50 feet of the apiary at Franklin and Marshall College in Lancaster, Pennsylvania. The apiary is located in a field near the Lancaster Country Day School and the baseball facilities of Franklin and Marshall College. There are also several organic garden plots located near the hive. At the time of this experiment, the apiary contained only one hive of bees.

Prior to training, cotton balls soaked in both mint-lemongrass and grapefruit essences were left out for the bees to visit. Few bees went to either scent, but those that came generally went to the grapefruit scent first. To ensure that a preference for the grapefruit scent did not contribute to positive results, the grapefruit essence was ultimately associated with the less valuable food source.
Training and testing were conducted throughout the month of September and the beginning of October in 2010. Training was conducted every day for 18 days unless it was raining; the bees were inactive on rainy days. Following the training period, testing was conducted over three consecutive days. The middle day of testing was overcast and cloudy, which caused a large decrease in the number of bees visiting the testing site, with only three bees visiting the testing site throughout the hour testing period. The data from this day was excluded from the final analysis as a result.

Prior to the training period, Petri dishes and Tupperware tops between five and 10 cm in diameter and containing 15 mL of 2:1 pure granulated sugar/water solution were left outside of the hive for a two-hour period for three consecutive days. This was done in order to acclimate the bees to the experimental set up and signal the dishes as a reliable food source. All pre-training was conducted in the morning between 09:00 and 11:00.

During the training period, the Petri dish containing the sugar water solution was left with a cotton ball soaked in approximately five mL of Mann Lake Ltd. ProHealth Feeding Stimulant, which was scented with mint-lemongrass essence. The cotton ball was placed in a second Petri dish next to the sugar water solution. The dish and cotton ball were left on a white plastic footstool that stood 23 cm off the ground, was 30 cm wide, and 33 cm long. All training occurred during the afternoon. A researcher sat on a green box next to the stool and marked all bees that visited the training station over a three-hour period. Bees were marked using non-toxic nail polish, and were marked on their lower abdomens. Green nail polish was used for the first phase of training. Piggy Paint brand nail polish was used, as it is non-toxic. Initially, the arrival time of the bee, whether it was previously marked, and the duration of its stay were recorded. Additional studies may wish to track the movement pattern of each bee during scent investigation and
feeding. During later observation periods, the number of bees became too great to record individual stay lengths, and instead the total number of bees, as well as the number of unmarked bees was recorded every minute, as the duration of each stay was approximately a minute long. The sugar solution in the dish was replenished throughout the testing period to ensure it was always a reliable food source for the bees. During food replacement, the bees did react to the experimenter, but not enough to leave the food source and not return. After the first week of training, sessions were reduced to an hour and a half, and bees were not marked within the last 15 minutes of testing, so only bees that were able to make repeated visits were marked. Training continued in this manner for 12 days total.

On the 13th day of training, two Petri dishes were set out using the same set up as was used during the training period. Both dishes contained 2:1 sugar solution, however, one dish was paired with the lemon mint smell while the other was paired with a novel smell, in this case, grapefruit essential oil manufactured by Aura Cacia. This was done in order to test if the bees were successfully trained to forage at dishes paired with the mint lemon scent. The test lasted 15 minutes, and the number of visits to each Petri dish was recorded over the 15 minutes. Video recording would have been useful at this stage in the experiment, however budget concerns prevented the use of recording equipment.

After the test was conducted, the novel smell was then used to train the bees to avoid a lower quality food source. The scent was paired with a Petri dish containing only water. Water was used as it has been shown to have a negative foraging value for bees, as is seen in other experiments to condition bees to avoid stimuli (Fischer et al. 1993). Additionally, previous studies have shown that negative associations to scents can be formed with both appetitive and aversive training paradigms, and negative associations to scents were formed with equal strength
using associations with water and with shock (Abramson 1986). The new scent and water dish were placed on the same stool as the positive scent association dish, but the dishes were placed in opposite corners. At the start of the second scent pairing, bees were re-marked with a different color to distinguish that they had been trained on the two scents. Marking and re-marking of bees did not seem to disrupt their behavior, as was seen in previous experiments that used nail polish or paint to mark bees during training (Fischer et al. 1993). Training sessions including both scents were conducted every day for a week. The total number of bees on each Petri dish was recorded every minute. The total number of bees visiting each dish for the first time was also marked. Both positive and negative associations were used in order to create an aversion to undesired foraging opportunities, and encourage foraging in desired locations.

The method used to train the bees was similar to the free flying method used commonly among bee researchers. However, the method was slightly different based upon location constraints. The apiary used in the experiment was not close enough to a lab to permit individual bee training in a lab setting. Instead, all bees were trained together in close proximity to the apiary. The bees were allowed to visit the training station freely, instead of being captured and transported to a training station in a lab. Also, although the bees were trained together, individual learning was still used.

To test the bees, I obtained two dozen cut orange and red chrysanthemums from the California Organic Flower Company. Three bunches were used. In order to simulate a non-organic flower, one bunch was sprayed with Ortho Ecosense Brand Garden Disease Control herbicide, with the active ingredient copper octanoate. The other two flowers were kept in organic condition. One organic bunch was sprayed with the mint lemon odor, while the other was sprayed with the negatively paired grapefruit scent. The organic flower/grapefruit essence
combination was used as a control to ensure the bees did not preferentially forage at one bunch because of the herbicide smell, or any other trait from the organic bunch. The non-organic bunch was sprayed with the grapefruit scent as well. Scents were applied by mixing them with about 50 mL of water to make them less viscous, and sprayed on the plants with a spray bottle for each scent. The scent was replenished daily to ensure it was detectable during each trial. The three bunches of flowers were placed in identical blue buckets with a metal and white handle for the testing sessions, and were placed in the same location as the training sessions. The buckets held a volume of 7.5 L.

Testing sessions were an hour each, and were conducted over three consecutive days immediately following the end of training. Due to weather restrictions on the second day as discussed above, the data collected from that day was not used in the analysis. A bee was considered to be on the bunch if it landed on the plant or on the bucket. The bucket was considered landing because bees often moved from the bucket to the flowers. The relative position of each bunch was changed every day to avoid any bias from the position of the bunch. Whether or not the bee was marked or unmarked was also recorded when possible. Recordings were made every minute.

Data were analyzed using t-tests to compare the positively-associated mint-lemongrass scent versus the negatively-associated grapefruit scents, as well as a repeated-measures ANOVA to compare the number of visits to each of the three groups of flowers. Tests were made for repeated measures, as differentiating between bees was impossible. T-tests were also conducted to determine if there was a difference in the number of bees visiting each scent during the training sessions.
The test was performed again in April 2011 to determine if the bees displayed retention of the training over different seasons. The same experimental set up was used, and the experiment was performed in the same locations. The only change in the experimental set up was daisies were used instead of chrysanthemums. This change was made based upon limits in flower availability due to season. Also, the markings made the previous fall had not lasted through the winter, so it was not known if the bees were previously trained.

Results

Training success was measured in two ways. The first test on preference for mint-lemongrass scent over the novel scent resulted in 43 visits to mint-lemongrass and 25 visits to the grapefruit. It took bees less than one minute to begin foraging at the trained scent, while it took six minutes for the first visit on the novel scent to occur. On first arrival to the test station, 41 bees visited the mint-lemongrass scent first, while two visited the grapefruit scent first.

The second test compared the total number of visits of trained and new bees to each learned scent. T-tests for the total number of bees visiting each Petri dish, and for the number of new bees visiting each Petri dish were performed. For the total number of bees, t=8.96, df=24, p<0.001, with significantly more bees visiting the mint-lemongrass scent. For the number of new bees visiting the training station, p<0.001, with more bees visiting the mint-lemongrass scent. The number of bees present at the training station generally increased over time, with the majority of bees foraging at the mint-lemongrass station (Figure 1).

During the first test day, 113 visits were made to the mint-lemongrass scented flowers, while the organic flowers sprayed with grapefruit had 42 visits, and the nonorganic flowers sprayed with grapefruit had 28 visits over an hour period. On the third day, only 37 visits were
made to the mint-lemongrass flowers, while the organic and nonorganic grapefruit scented flowers had 15 visits and 11 visits, respectively. The total visits to each treatment group were then 150, 56, and 39 to the mint-lemongrass scent, the organic grapefruit scent, and the nonorganic grapefruit scent, respectively (Figure 2). Significantly more bees visited the mint-lemongrass treated flowers on both days one and three (p< 0.001 for both tests). For the total number of visits over each test day, f=41.467, df=2, p<0.001 as well.

Pairwise comparisons for each group on day one of testing indicated a significantly different number of bees visited the mint-lemongrass station than either station treated with the grapefruit scent (p<0.001). The number of visits between the two grapefruit treated plants was not significantly different on day three (p=0.454). However, on day one and for all days combined, it was statistically suggestive that the bees foraged more often at the organic grapefruit treated bunch (p=0.070 and p=0.055, respectively).

During the re-test of the foraging population in the spring, only three bees visited the testing station. One bee landed on each of the test conditions. Several other bees flew over the testing station, but did not land and thus were not recorded.

**Discussion**

During both the training and testing period, the bees chose to forage at the positively-associated mint-lemongrass scent significantly more times than they foraged at the negatively-associated grapefruit scent (Fig. 1 and 2). This indicates that they were able to learn the relationship between the mint-lemongrass scent and a productive food source. The bees’ ability to learn a relationship between a specific scent cue and a reliable food source has been well established in the literature (Bhagavan and Smith 1996), but the testing situation placed the
learned association in a novel context. In the test situation, the bees were faced with foraging options that were identical, except for the odor associated with each option. Since the bees still foraged significantly more times at the scent that signaled a reliable food source (Fig. 2), it shows they can retain and transfer associations between different contexts. Transfer of learning occurs when old or new stimuli are presented in new context, or the ability to categorize stimuli is seen in an organism (Bouton 1994). Positive conditioning has been shown to be generally successful in vertebrates, but is untested in bees (Bouton 1994). Context information like time or experimental traits can help organisms guide retrieval of learning, and plays an especially large role in phenomena like extinction and counter conditioning (Bouton 1993). However, when stimuli are transferred to new contexts, the retrieval of training may not be as effective, and performance of a learned behavior may not be perfect in a new context (Lovibond et al. 1984). The ability of the bees to transfer training to a novel context indicates that the training was successful for the stimuli, but not the context, so it is likely more practical on a wide scale.

However, because each foraging condition received one visit from a bee during the spring retest, there was no evidence the bees retained training over the seven-month interim. It is possible the retest was performed too late in the spring, so that the individuals trained during the fall were no longer alive and foraging (honey bees generally only live for 6 or 7 months over the winter). If this experiment were performed again, it would be interesting to investigate whether there is a difference in behavior from early March to April as the foraging population begins to change. Additionally, it would be interesting to see if retention occurred if the same flowers were used, as opposed to a different species.

The results of the training session also indicate that there could be some communication within the hive concerning the reliability of the mint-lemongrass odor. The number of new bees
visiting the mint-lemongrass scent was significantly greater than the number of bees visiting the grapefruit scent (Figure 1). Foragers that had never been exposed to the training session would be expected to investigate both scents on their first visit, especially when the spatial location of the productive food source changed with each trial. However, only a few bees investigated the grapefruit scent on their first visit to the training station, so there could have been some communication within the hive concerning the reliability of each scent to signal a productive food source. However, it is likely that this communication within the hive was scent based, and not due to a communication between hive members about training. Bees communicate information about food quality and identity through both nectar smell, and the residual flower smell on a foraging bee (Gil and DeMarco 2005), so it is likely the bees bringing back the mint lemongrass smell from training influenced the foraging activity of bees that had never experienced training. More research into this area is necessary in order to make more concrete conclusions.

In addition to demonstrating the sophisticated learning abilities of honey bees, these results have many practical applications. The bees in this experiment were trained to avoid a non-organic food source that could present a potential threat to their health. If bees can avoid plants that are treated with synthetic chemicals, than the success of the hive might be improved (Decourtye et al. 2005). Training the bees to only forage at some food sources can help with this avoidance, and ensure honey bees forage at plants that are not detrimental to their overall health. This, in turn, may alleviate some of the impacts of other diseases and make honey bee populations stronger. However, the pairwise comparisons of the number of visits each minute to the test stations showed that there could be some natural aversion to a plant that was chemically treated. While there were no statistically significant differences between the two grapefruit
treated plants, the p values of 0.055 for the total grouped data and the p value of 0.070 for the Day 1 data suggest that there could be a difference between these two conditions. The reasons for this difference are not known, and should be researched further. Because there was no evidence of training retention between seasons, however, in order for these results to be applied in a real world context, the ability of the bees to relearn this information, and how quickly they can relearn the training must be researched.

There were also several interesting factors associated with the training and testing process. Both factors are related to the idea that bees can learn associations between colors and a food reward, as well as odor associations (Bhagavan and Smith 1996). All training sessions were conducted in close proximity to the car used to transport all bee keeping and training materials, including the sugar water used for training. Through the course of training, an increasing number of bees marked with nail polish would approach the car as it pulled up to the training site and sit in the trunk during experimental set up, indicating that the bees could also have associated the car with a food reward, mostly likely through a color association. For this reason, the car was not parked in close proximity to the test station on test days, as it may have influenced the results of the experiment. During the test days, there was also some tendency for the bees to land on the white handle of the bucket before landing on the flowers. It is possible then that the bees could also have associated the white color of the stool used in training with a food reward, and were then attracted to it during the test phase of the experiment. However, this probably did not affect the results of the test. Previous research has indicated that bees can exhibit overshadowing, in which less conditioning occurs to a weaker stimulus if it is paired with a strong stimulus (Bitterman 1996). In this case, the color white would have been paired with both the positive and negative reward, making it an unreliable, and therefore weaker stimulus. The scent would have
been a more reliable stimuli, so the bees would have paid more attention to scent then color. Because color was a weak stimulus, and used for both the positive and negative training systems, it would probably not impact the preferences of the bees.

This study can serve as a valuable starting point for many future studies. First, this study shows that bees can show learned preferences for a learned cue in a field setting. In most honey bee learning experiments, single bees are trained and preferences are measured using choice of foraging location, or proboscis extension in response to a cue. In this experiment, the entire foraging population of the hive was trained to respond to an odor cue at the same time. This learning was then tested both with the traditional set up using sugar water and then in a completely new context, demonstrating that the bees were capable of transferring learning across contexts. This study could also lead to future research into helping bees avoid unhealthy food options, such as plants that have been treated with pesticides. An ideal area for future research would include testing if this behavior can be seen across a wider geographic scale, or if it can be maintained in a different spring when more foraging options are available. The lack of retention of training seen in this experiment indicates that experiments would have to include repeated training sessions. Future research could also focus on the length of training retention, and if this length is long enough to permit use in a real world context.
Works Cited


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Figure 1. The number of bees visiting each testing station at five-minute increments. The lines are divided both into the number of new bees visiting the testing station and the total number of bees at each condition in the testing station. Results were obtained from counting the number of bees at the test station every five minutes.
Figure 2. The total number of bee visits to each test condition over an hour period for both test days combined. A visit was counted as any bee landing on a flower or on the bucket for each condition. Recordings of bee visits were made by counting the number of bees on each treatment every minute.