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The Analysis of the Effects of Smoke and Water Vapor on Insect Pheromone Communication and Physical Condition: An Investigation of the Causes of Colony Collapse Disorder

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Summary

Since the outbreak of honey bee (*Apis mellifera L.*) disappearance in the phenomenon known as colony collapse disorder (CCD) in 2006, investigations dedicated to finding the causes of CCD have suggested possible factors; however, a singular cause has not yet been identified. This project aims at identifying how external factors in the environment, such as water vapor and smoke, can affect the social behavior and physical condition of honey bees. It was hypothesized that water vapor could block insect pheromone communication and that smoke could accumulate on the antennae of insects, interrupting pheromone communication. House crickets (*Acheta domesticus*) were used to test water vapor's effect on pheromone communication because they communicate with pheromones like honey bees. Crickets were exposed to vinegar, mimicking the alarm pheromone, to confirm a social response. Next, we tested water vapor's ability to block pheromone communication. To test the effect of smoke's presence in the environment, red harvester ants (*Pogonomyrmex barbatus*) were used, due to their anatomical similarity to bees. The red harvester ant experiments measured the degree to which smoke particles accumulated on their body parts. The results showed that smoke particles did accumulate during exposure to smoke. This study provides evidence that external factors in the environment of insects may act as pheromone blocking agents and thus obstruct the insects' ability to communicate through pheromone messages. This conclusion may provide an explanation for the observations in CCD such as the honey bee disappearance and diminishing health.

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Introduction

Many research projects have been dedicated to finding the cause of colony collapse disorder (CCD) in honey bees; however, no singular cause for the disorder has been found. There have only been likely causes identified rather than a conclusive cause or combination of causes. Bees are an integral part of the pollination of plants. They help pollinate about 80% of the world's plants (1). The outbreak of CCD in 2006 threatened the ecosystems of plants that require pollination through bees, which in turn affected the U.S. agriculture industry. If not stopped, the disorder may result in further endangerment of the honey bee, drops in crop production, as well as a loss in the balance of many ecosystems due to lack of sufficient pollination (2).

Since 2006, beekeepers and scientists have been studying CCD. Part of the unsolved mystery of the disorder is that the bees disappear but are not found dead. In the winter of 2006-2007, the death rates in honey bee hives ranged from 30% to 90% (3). The symptoms of CCD have been observed up to one year before the colony collapses and include lowered honey production and fewer bees in the colony. In the later stages, up to six months before collapse, brood nests are slow at expanding and they are left behind after adult bees emerge. The population fluctuates in numbers and honey production remains level during the growing season. Three months before collapse, bees often form soft ball–sized clusters, like those made in the winter, and try to recover from population loss. In the final stages of colony collapse disorder, the queen of the hive lays as many eggs as possible, so as to recover the population; however, workers are not returning to the hive with nectar and pollen, so the source of food drops. The combination of these forces results in the starvation of the larvae, and the colony soon collapses (4).

There are many possible causes that have been linked to colony collapse disorder, such as decreased immune strength, pathogens from external sources, and pesticides in the environment. Varroa mite infestations and pesticides, such as neonicotinoids and imidacloprid, have been detrimental to bee health (5). Investigation has shown that imidacloprid exposure results in symptoms similar to CCD (6). Governments have set up foundations to monitor the changes in CCD; however, bee colonies continue to decline and a definitive cause has not been found.

This project investigated how external factors in the environment, such as water vapor and smoke, can affect

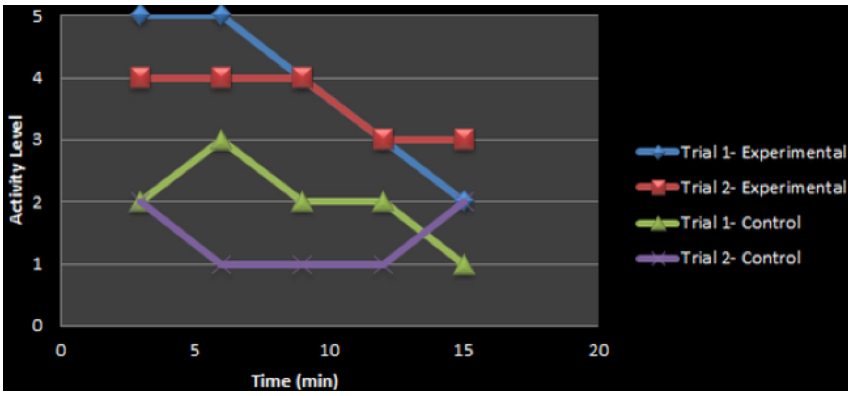

Figure 1: House Cricket Social Behavior in the Presence of Volatile Acetic Acid. The activity level changes for each trial is shown. Group 1 was used for Trial 1 Experimental and Trial 2 Control. Group 2 was used for Trial 2 Experimental and Trial 1 Control. Experiment was done at 22 °C.

insect pheromone communication and physical condition. It has been observed that flying insects, including bees, are sometimes hindered by humid weather and are not as adept at flying as they are in dry weather (7). This brought us to the hypothesis that water vapor may cause a break in pheromone communication. Since access to a working honey bee hive was not a possibility, house crickets were used as a substitute (*Acheta domesticus*). Crickets are anatomically similar to honey bees with the same body parts, specifically antennae, head, thorax, abdomen, and legs (8). They also communicate via semiochemical signals, which create pheromone alarm social responses. The alarm pheromones of honey bees, including isopentyl acetate and 3me-2-butenyl acetate, are volatile, low-mass molecules that easily diffuse through the air as an alarm signal. One of the primary components of cricket alarm pheromone is acetic acid; therefore, vinegar was used to simulate the alarm pheromone in the cricket experiments. Vinegar is also volatile, low in molecular mass, and able to transmit a signal (9, 10). It was hypothesized that water vapor would inhibit pheromone communication in crickets, due to a masking of the pheromone scent by the water vapor. The experimental results supported this hypothesis. There was less of an alarm pheromone social response when water vapor was present.

Since the smoking of bees is a commonplace practice used by beekeepers, it was hypothesized that excessive use of this practice could be another environmental factor contributing to colony collapse disorder. An accumulation of smoke particles could potentially cause a blockage of antennae receptors, which are important to semiochemical communication. Red harvester ants *(Pogonomyrex barbatus*) were used for this phase of experimentation because of their ability to communicate via pheromone messages. Ants use many different forms of semiochemical communication such as with alarm, trail, and recruitment pheromones (11). It was hypothesized that regular exposure to smoke would cause it to accumulate on the surfaces of the ants' bodies. The experiment with ants tested the accumulation of smoke on their bodies, but it did not directly test if this effected pheromone communication.

Results

The goal of this research was to determine the relationships between the social response of insects similar to honey bees and the external environmental factors of water vapor and smoke. The first two experiments were aimed at finding the relationship between the presence of water vapor and alarm pheromone response in house crickets. We tested whether an alarm response could be induced by exposure to volatile acetic acid (household vinegar),

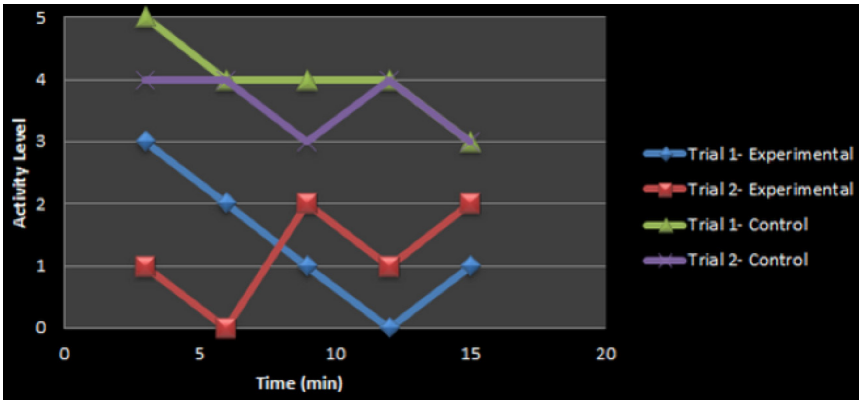

Figure 2: House Cricket Social Behavior in the Presence of Volatile Acetic Acid and Water Vapor. The activity level changes for each trial are shown. Group 1 of crickets was used for Trial 1 Experimental and Trial 2 Control. Group 2 of crickets was used for Trial 2 Experimental and Trial 1 Control. Experiment was done at 22°C.

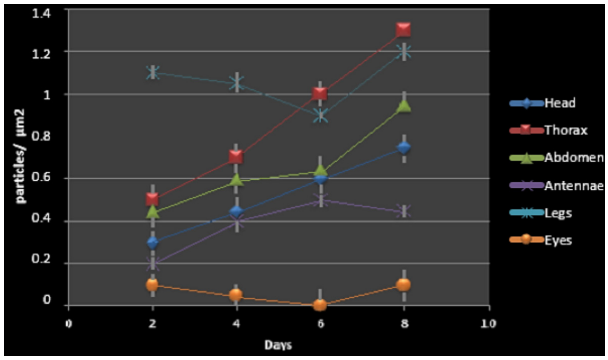

Figure 3: Smoke particle abundance on experimental red harvester ants over time. The average levels of smoke particle abundance on several parts of the body in the experimental ant group is shown. The error bars represent the standard deviation of the collected data.

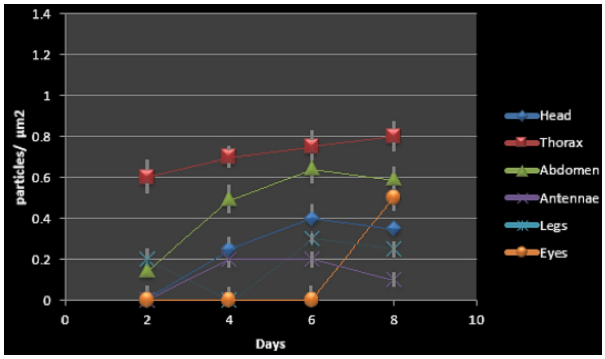

Figure 4: Smoke particle abundance on control red harvester ants over time. The average levels of smoke particle abundance on several parts of the body in the control group is shown. The error bars represent the standard deviation of the collected data.

which is a component of an alarm pheromone. After confirming this response, experiments were conducted to see if water vapor could alter or block this response. Experiment 1 (**Figure 1**) tested the prompting of an alarm pheromone reaction using volatile acetic acid. The crickets were divided into two groups of ten each. The control group was not exposed to any outside trigger and the experimental group was exposed to volatile acetic acid. Each trial lasted for a period of 15 minutes, and the alarm pheromone response was measured according to a 0–5 scale for activity, as specified in the Materials and Methods section. Each trial had an experimental group and a control group. There were two trials, and in order to ensure the group itself was not simply more active, they switched between the role of the control and experimental group.

This experiment tested the relationship between the prompt of the alarm pheromone (volatile acetic acid) and the social behavior of the crickets. As seen in **Figure 1**, the activity levels in the experimental groups are higher than those of the control groups. The experimental groups experienced a downward trend over time, suggesting that the volatile acetic acid escaped from the container, thereby causing a decrease in the alarm pheromone response. The control group experienced a change in behavior that was significantly less than that of the experimental group. The results of this first experiment provided evidence that the acetic acid does, in fact, trigger an alarm response in the house crickets.

With this relationship defined, Experiment 2 was conducted to test the ability of the water vapor to block the alarm pheromone social response. It was expected that the water vapor could interfere with the pheromone communication system (artificially prompted as in Experiment 1). During Experiment 2 the control groups were exposed to volatile acetic acid alone, while the experimental group was exposed to acetic acid in the presence of water vapor. The crickets were in larger containers and the amount of acetic acid was increased proportionally. Experiment 2 consisted of two trials, and the control group and experimental group of ants were switched between the trials, just as in experiment 1.

As seen by observing the changes in activity levels in the house crickets that were treated with both volatile acetic acid and water vapor (**Figure 2**), it can be concluded that the alarm pheromone social response decreased when the water vapor was present. The activity in the control group of this experiment was consistent with that of the crickets exposed to volatile acetic acid in Experiment 1. The crickets had a high activity level when there was acetic acid in the air of their container. The activity of the house crickets treated with both acetic acid and water vapor was lower, suggesting that the alarm pheromone social response was by blocked by the water vapor.

In the Experiment 3 which followed, it was predicted that the smoke sources in the environment, including smoke from the common practice of smoking bees to control them and smoke from pollution, could be harmful to insects. This study tested whether or not smoke accumulates on the surfaces of insect bodies. To do this, red harvester ants were used and split into an experimental group and a control group. For the first part of the experiment (**Figures 3 and 4**) the experimental group was given three doses of smoke per day for a total of eight days. The smoke was created by burning paper, leaves, and wood: all sources of smoke in the environment that are created by bee smokers or pollution. The ants' bodies were analyzed under a microscope every two days. The smoke particle abundance was measured for different parts of the bodies of the ants. These observations were made approximately three hours after each smoke application.

Over the period of eight days of observation, the smoke particle abundance increased in most parts of the ants' bodies in the experimental group (**Figure 3**).

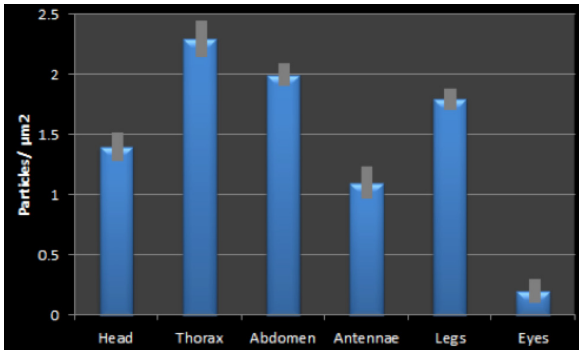

Figure 5: Average values of smoke residue abundance directly after dose (particles/micrometer squared). The average values of smoke particle abundance for ants observed directly after being dosed are shown. The error bars represent the standard deviation of the collected data.

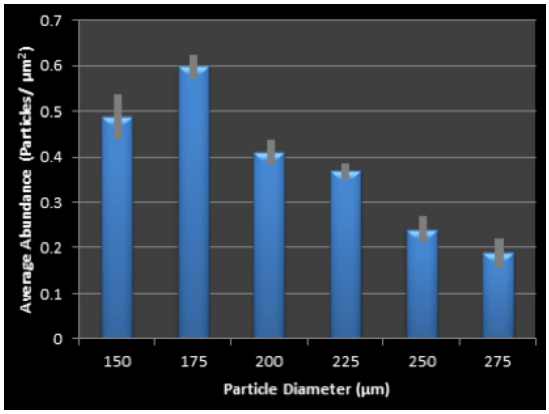

Figure 6: Abundance of various sizes of smoke particles. The average abundances of particles of different diameters is shown. The error bars represent the standard deviation of the collected data.

The trends present in the experimental group were visible in almost all body parts with the exception of the ants' eyes and legs. The changes over time in smoke particle abundance did not occur at the same rate for all the parts of the body observed. This is in part because of ant behavior, such as the ants rubbing their antennae after the smoke dose was given. This suggests that the ants removed some of the smoke particles themselves. The control ants did have some smoke particles on the surfaces of their body parts; however, they were not as high as the levels in the experimental group (**Figure 4**). They also did not change over time.

Because previous observations had been taken up to four hours after the smoke doses had been given, a separate experiment was done to determine the smoke particle abundance directly after the smoke dose was given. It was expected that this would give a more accurate picture of how smoke sticks to the surfaces of ants' bodies without the factor of the ants trying to clean the smoke particles from their bodies. This was tested in Experiment 4 (**Figure 5**). Ants were given a smoke dose and then the smoke particle abundances on their bodies were observed directly after the dose was given.

The smoke particle abundance levels measured directly after the smoke dose show higher levels than in the experimental group's last measurements (Experiment 3) on most parts of the body. The most interesting part of the data is that the antennae had a much higher level of smoke particles present directly after the dose. This confirms that it was the ants themselves that had, with their own legs, removed some of the smoke particles from the antennae. The smoke particle abundance levels in the eyes, however, were not changed, suggesting that the smoke particles do not easily stick to the surface of the eyes. In addition to this test, the abundance of various sizes of smoke particles was found. This was done by taking the average abundance of particles that were binned by size. The abundances of the different sized particles were measured and it was found that the most abundantly-visible particles were of 175 micrometers in diameter.

Discussion

This study aimed to find relationships between the presence of external factors, such as water vapor and smoke, and the ability of insects to communicate via pheromones. The results of the test of water vapor's effect with the house crickets pointed towards the conclusion that water vapor can obstruct house cricket pheromone communication. The water vapor appeared to reduce the house cricket alarm response by blocking the volatile acetic acid molecules from being received by the crickets. The crickets in the experimental group in Experiment 1 showed an alarm pheromone social response when they were treated with volatile acetic acid. They ran around the container and tried to escape from it. Alternatively, the control group did not act differently than normal behavior, confirming that the acetic acid really did prompt the response. With this relationship established, water vapor's effect on the pheromone communication could be tested. The results of this experiment (Experiment 2) confirmed what had been hypothesized: the pheromone prompt was much less effective with the water vapor present. The levels of activity for the experimental group in Experiment 1 (**Figure 1**) had been high, confirming that the social response had been prompted. In Experiment 2 (**Figure 2**), however, the activity levels were much lower in the alarm social response with water vapor present.

When testing smoke's effect, the microscope images showed how smoke particles accumulated on the surfaces of the ants' bodies over time. This confirms what was hypothesized. The smoke particle abundances of the ants treated with smoke trended upwards during the experimentation period (Experiment 3, **Figures 3**

5	Very High Activity: All the crickets run around the container and try to escape. All crickets are moving quickly and their antennae are moving around.
4	High Activity: The house crickets move around in the container constantly and try to climb the walls to escape.
3	Moderate activity: The crickets move often and some try to escape the container.
2	Some Activity: The crickets walk slowly; however, none try to climb the walls to escape.
1	Low activity: The crickets move sometimes. They are all separate and do not interact.
0	Very Low Activity: The crickets are only moving between several second intervals. They are otherwise completely still.

Table 1: Cricket activity scale.

and 4). The control ants did not show such trends. When it came to comparing the trends of different parts of the ants' body in the experimental group, however, there was more of a surprise. For the antennae of the experimental group, there was a trend upwards in the accumulation of the smoke particles; however, it was not as steep a trend as other parts of the body. It was observed that the ants would rub on their antennae after the smoke doses with their two front legs. This is considered the reason why the trends for the antennae are not as steep, as they would clean some of the particles off themselves. In other body parts, such as the eyes of the ants, there was almost no accumulation in both the experimental group and the control group. The body parts with the sharpest trends in smoke particle accumulation included the thorax and the legs. In the control experiment, there were some particles of dust on the surfaces of the ants' bodies; however, they were at significantly lower levels than the experimental group, and they remained static over time. In addition to observing the smoke particle abundances, the particle radii were measured (**Figure 6**). The most abundant particles visible through the microscope were those with a radius of approximately 150-175 μm. The particle sizes of least abundance lay in the 250-300 μm range. Knowing the particle sizes may prove useful in making future hypotheses about how smoke may affect insects anatomically.

The materials and methods used were designed to maximize accuracy and precision of the data collection, but some limitations did exist. The number of crickets used in each trial varied because some of them died during the timeframe of the experiment. However, regardless of the number of crickets used, the patterns viewed were the same during each trial. Another possible limitation is the substitute for cricket pheromone; if all the cricket alarm pheromone components had been used, there might have been a fuller alarm pheromone response. The full pheromone might create bigger and more significant differences in activity levels in the different trials. The limiting factors in experimentation with ants include the fact that the microscope was only able to view particles of 150 micrometers or larger. This means that there could have been particles less than 150 micrometers affecting the ants in a significant way.

The results of the investigation support the hypotheses of both projects. The smoke builds up at a slow rate on the bodies of the insects, and the water vapor can cause difficulties in pheromone communication between insects. These effects may help to identify factors that are causing colony collapse disorder. Though they might not be the direct or singular causes of CCD, they are possible explanations for the unanswered questions made by scientists and beekeepers. For example, long periods of a humid climate in a region may inhibit honey bees and other insects from communicating through pheromones. Since smoking is a standard method used by beekeepers when controlling a hive, it is also likely that smoke particles adhere to bees' bodies in the same way that they adhere to ant's bodies. It is also possible that the smoke irritation has a detrimental effect on the health of the bees in the hive. Future experiments should attempt to address these questions.

Materials and Methods

Experiments with crickets

The house crickets (*Acheta domesticus*) were kept in an Uncle Milton brand "Bug Jug." They were kept indoors near a window at temperatures of 21–24°C. The temperatures were kept in these ranges to ensure stable conditions that were similar to those of the crickets' natural habitat. They were fed Fluker's brand cricket food, as well as carrots. The food was placed in a petri dish in the amount of 2 tablespoons and was replaced every four days. Two carrots were fed at a time and replaced every three days. The crickets were given water every day on moistened cotton balls. The containers were cleaned every two days, and fecal matter was washed from the bottom of container using Dawn dish soap. Dead crickets were removed immediately, in order to avoid diseases or fungal infections spreading to the other crickets. The equipment used for the experimentation included a Thermometer-Enviro-safe by H.B. U.S.A (-20°C–150°C), a Mass- Philips-Essence HR 2394 (measures in grams up to 5000 g), a Container-Rubbermaid-401C 25, Distilled Vinegar from Grain or Corn, Stop and Shop Brand (5% Acetic Acid 0.83 M solution); a spray bottle,

and a timer.

Determining the effect of the pheromone component on crickets

Volatile acetic acid is a component of a cricket alarm pheromone; therefore, household vinegar was used during this phase of experimentation as the alarm response prompter. Ten crickets were placed in two separate15 x 5 x 15 cm glass containers. The temperature and the pressure of the room were recorded. Five milliliters of the acetic acid solution were measured using a syringe and then it was quickly put into the container. The cricket's behavior was observed for 15 minutes, rating the activity every 2 minutes. The behavior was measured from a 0–5 scale with the specifications for each activity level shown in **Table 1**.

The control group was observed with no acetic acid solution in the container. The cricket groups were alternated between the control and the experimental containers, and each container was washed with Dawn dish soap between the trials. Between trials, crickets that were not being used were kept in the "Bug Jug."

The effect of water vapor on pheromone communication

Eight crickets were placed in a glass jar. The temperature was read and recorded. Twenty-five milliliters of acetic acid solution were placed in a petri dish at the bottom of the jar, then three sprays of water was sprayed 1.5 meters above it. The mist was allowed to fall into the jar. The jar was then closed and observations were made for ten minutes. Acetic acid was applied to the control group without the presence of the water vapor. Each trial was conducted twice so as to ensure repeatability.

Experimentation with ants

The red harvester ants were housed in two separate glass containers (with holes and transparent covers) near a window. They were fed equal amounts of food and water during an acclimation period of one week. They were given a small piece of bread (Stop and Shop Brand Italian) with the crust included. It weighed about 5.0 grams. The bread in the containers was changed every three days. The water was given to them by wetting a small piece of tissue with deionized water and then wringing it out slightly to avoid puddles that might cause the ants to drown. After the first week, the ants were moved to a plastic container, kept near a window, and monitored at a steady temperature of 22°C.

After the acclimation period, the ants were tested over a period of one week. The control group was fed normally and left alone without external influence. The experimental group was treated with doses of smoke inside the housing container. The smoke was produced by burning printer paper, wood, and dead leaves from the forest. The flammable materials were rolled up, ignited, and then blown out, creating smoke that was wafted into the container. This procedure was done three times a day: in the morning (6:30 a.m. – 7:00 a.m.) and again around noon and at night (8:00 p.m. – 9:00 p.m.). The smoke was left in the container for one minute (30 seconds wafting it in and 30 seconds with another cover keeping it in). After the smoke was released, residue from the smoke, such as larger pieces of ash, was removed from the container with dry paper towels. After smoking, the lid was replaced. Small holes allowed for air circulation. Approximately three hours after the second application of smoke, the ants were removed from the container and observed under a microscope that could detect particles of 150 micrometers or greater. The particle abundances were measured for each body part by averaging five 100 square micrometer samples of smoke particle abundances. The number of smoke particles in each sample was counted for each of the body parts to get the values that are graphed. This was done for one of the ants in each group each time observations were taken (every two days).

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References

1. Mori, Scott. "Pollination." Pollination. The Encyclopedia of the Earth, 31 Mar. 2009. Web. 5 Nov. 2013.
2. "Biology of Plants: Pollination." Biology of Plants: Pollination. Missouri Botanical Garden, n.d. Web. 08 Nov. 2013.
3. CCD Steering Committee. Colony Collapse Disorder Report. Rep. N.P.: N.P., 2012. Print.
4. Bromenshenk, Oliver, and Westervelt Debnam. "Bee Culture." Bee Culture. N.P., 1 Feb. 2009. Web. 17 Feb. 2014.
5. Yang, X. and D.L. Cox-Foster 2005. Impact of an ectoparasite on the immunity and pathology of an invertebrate: Evidence for host immunosuppression and viral amplification. *Proc. Natl. Acad. Sci.* USA. 102: 7470-7475
6. Lu, Chengsheng. "In Situ Replication of Honey Bee Colony Collapse Disorder." Harvard School of Public Health. Bulletin of Insectology, 13 Mar. 2012. Web. 15

Nov. 2013.
7. Oles, Ken. “Colony Collapse Disorder in New England.” Research and Innovation Class. King Philip Regional High School, Wrentham, MA. 4 Feb. 2014. Lecture.
8. “Honey Bee Pheromones Are an Integral Part of Their Communication System.” Honey Bee Pheromones Are an Integral Part of Their Communication System. Keeping-Honey-Bees, n.d. Web. 03 June 2014.
9. Hunt, G. H. Semiochemicals of Apis Mellifera. Publication. Pherobase, n.d. Web.
10. El-Sayed, Ashrad. “Semiochemicals of Acheta Domesticus, the House Cricket.” The Pherobase. N.p., n.d. Web. 10 Feb. 2014. <http://pherobase.com/database/species/species-Acheta-domesticus.php>.
11. Liu,Y.,and J. L. Benthuysen. “Pheromones and Semiochemicals of Pogonomyrmex Barbatus.” Pheromones and Semiochemicals of Pogonomyrmex Barbatus. Pherobase, n.d. Web. 30 Apr. 2014.