The Effect of Sulfur Dioxide on the Olfactory Learning of Western Honeybees (*Apis mellifera*)

Soyoun Moon, Lakxshanna Raveendran

Commack High School

ABSTRACT

Air pollution is a significant problem within our environment as the continuous burning of fossil fuels has resulted in the production of pollutants such as sulfur dioxide (SO2). Pollution poses a threat to Western Honeybees (Apis mellifera) as they reduce honeybees' ability to perceive floral volatiles and airborne pheromones, thereby effecting their foraging efficiency. Knowledge of the importance of olfactory learning in young bees is imperative to research as nearly 73% of cultivated crop varieties are pollinated by bees. The objective of our project was to learn about the effects of sulfur dioxide on the olfactory learning of foraging naïve bees and analyze the severity of limonene degradation in New York. Honeybees were classically conditioned to lavender using the Proboscis Extension Reflex (PER) Assays. If the Honeybee extended their proboscis after lavender exposure and before the sugar reward, we considered the assay to be responsive. A total of 0.03 moles of sulfur dioxide was produced, which the experimental group was exposed to for approximately 3 seconds before conditioning. Our results showed that the control group responded by extending their proboscis an average of 67.92% of the trials while the experimental group responded for 18.33% of the trials. This data supports the conclusion that sulfur dioxide increases the time of olfactory learning in foraging naïve honeybees. A choropleth map of New York was created with computational analysis where each county was shown in one of five colors, the darkest color being the greatest level of degradation of limonene by diesel exhaust, a common floral volatile and a major source of sulfur dioxide.

INTRODUCTION

The burning of fossil fuels has led to increasing amounts of pollutants being produced, which threatens the well-being of natural habitats, wild animals and humans. Though many nations are now enforcing laws to regulate these rates of pollution egression into our atmosphere, it is crucial to be aware of the effects pollutants will have on our environment.

The levels of sulfur dioxide, a common air pollutant in our society, depend directly on human actions. Pollution in the habitats of endangered species can be especially harmful as they can support their decline. The increase in air pollution poses a threat to the population of Western honeybees (*Apis mellifera*), as studies show that air pollutants reduce their ability to perceive floral volatiles, preventing pollination (Ginevan et al., 1980). Honeybees use airborne pheromones to find their food sources such as nectar and pollen. Pollutants disrupt this process, as they deteriorate these pheromones thereby increasing difficulty of finding flowers to pollinate. These pheromones also help honeybees to distinguish previously depleted flowers to maximize foraging efficiency (Slessor et al., 2005). As a result, it is imperative to research the effects of potentially harmful pollutants such SO_2 to honeybees in order to gain knowledge of their dangers and understand how the issue of the honeybee decline can be addressed.

We hypothesized that if honeybees are exposed to SO_2 before classical conditioning, it will take them a longer time to associate the lavender scent as a floral source. We also hypothesize that if honeybees are exposed to SO_2 after classical conditioning, the honeybees will no longer extend their proboscis as SO_2 will impact their olfactory memory (Bitterman et al., 1983).

MATERIALS AND METHODS

When classically conditioning the bees, the following methods were used. We left the honeybee in a freezer for 5 minutes to serve as a form of anesthesia. If the honeybee was still active, we left it in the freezer for an additional 1-2 minutes. Once the bee was inactive, we harnessed the bee in a modified bullet using a thin piece of tape. We placed the bee such that its head leaned over the edge of the bullet tip, allowing the thorax to rest on the inner ledge. Once these preparations were finished and the bee was recovered, we began the conditioning. First, we exposed the honeybee to 2 mL of lavender oil. The lavender oil was pipetted onto a strip of filter paper. The filter paper was then secured to the plunger seal of a syringe using a thumbtack. We pushed the plunger of the syringe inwards, expelling air towards the bee. The lavender oil was followed by rewarding the bee with a 10% sucrose solution. The reward was given by applying 2 mL of the sucrose solution on the tip cotton swab then gently tapping the antennae of the honeybee with the swab. In between the period of exposing the bee to lavender and providing the reward, the PER assay was conducted.

In our study, the Proboscis Extension Reflex (PER) Assay was used to determine whether the floral volatile prompted the honeybee to respond. We observed and scored the extension of the proboscis. If the honeybee extended its proboscis during the lavender but before the sucrose a '1' was recorded (responsive). If the proboscis did not extend to the lavender '0' was recorded (unresponsive).

To create the sulfur dioxide, we covered the bottom of a 2 necked round bottom flask with 10 grams of sodium sulfite, measured using a weigh boat. Then we added 6M of sulfuric acid to the 2 necked round bottom flask, which allowed the pressure-equalizing dropper addition funnel to slowly drip in by slightly opening the stopcock's flow. After, we opened the stopcock

connected to the main flask and rubber hose once the reaction began. The sulfur dioxide travelled through the rubber hose, making the dry gas available for our experiment.

For our experimental trials, we exposed the honeybees to approximately 3 seconds of sulfur dioxide gas. After this exposure, we conducted our classical conditioning procedure which included PER assays with 15 trials. We conducted the classical conditioning procedure for the control group likewise, but without the exposing the honeybees to sulfur dioxide.

To ensure that honeybees were conditioned solely to the scent of lavender, an ABABBA test was used. A represented lavender oil scent while B represented banana oil scent. After the experimental and control trials were conducted, we exposed the honeybees to said scents in the ABABBA order. The honeybees should have only extended their proboscis to the lavender scent to confirm that they responded to only the lavender stimuli. The bees considered in our data collection only responded to lavender oil scent in these tests.

For the computational analysis portion of the project we imported the National Emissions Inventory (NEI) source type data as a CSV (comma separated value) file. Then we imported libraries including: NumPy, Pandas, and Plotly.tools. After we created empty arrays for black carbon, sulfur dioxide, nitrates, and carbon monoxide, and FIPS County numbers. We created a for loop such that as the program reads through the data, for each county in New York, the total emission tons of black carbon, sulfur dioxide, nitrates, and carbon monoxide from mobile source types will be added. The sum of these separate mobile source type pollutant emissions represented the amount of diesel exhaust (tons) emitted per year in each county. The totals were multiplied by 1,000 to convert emission tons to liters. The totals were also then multiplied by 0.4 to represent the degradation of Limonene, a floral volatile commonly used by pollinators. For every liter of diesel exhaust there was in the environment, there was a loss of 0.4 in the

concentration of limonene. These new values were listed under the name "calculated". These new values represented the total loss of limonene concentration within a year in each county. A scale of degradation from 1-5 was created by dividing the range of degradation between the counties by 5. The counties were then given a value of 1-5 based on the severity of degradation there. The Plot.ly FF function was used to create a choropleth map of New York where each county was shown in one of five colors, the darkest color being the greatest level of degradation, the lightest being the least level of degradation. A legend was included showing the range of values included within each color value.

It is important to acknowledge the possible risks of our experiment. Be aware of the chemical used as sulfur dioxide causes severe skin burns, eye damage and is toxic when inhaled. Be cautious when handling the bees as well. To prevent any injuries or harm we wore protective gloves, goggles and a mask, as well as working under a fume hood in a well-ventilated area. We were also cautious when handling honeybees, as they have the potential to sting. For disposal, we allowed for the sulfur dioxide to burn off. Any residual waste was returned to the waste container.

RESULTS

It was hypothesized that if honeybees were exposed to sulfur dioxide before classical conditioning to a floral volatile, olfactory learning would take longer due to an impairment of their olfactory systems. According to the graphs and the data collected, this hypothesis was supported. Our data indicated that sulfur dioxide significantly impacted the olfactory learning of the honeybees within our experimental group. The average percentage that the bees responded to the PER assay were 67.92%(control) and 18.33%(experimental) respectively. Additionally, when comparing these percentages, the standard deviations, 7.5(control) and 7.4 (experimental)

showed no overlap in the error bars, suggesting that there is a significant difference between the control group's and experimental group's results. This was verified by a T-test as the p-value (1.00758E-35) was less than 5%. ABABBA testing also indicated that the honeybees were only trained to lavender in both groups.

Table 1: Responsive and Unresponsive PER Assays in Control Group.

(1=Responsive Assay; 0=Unresponsive Assay)

	Trials																
Bee #	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	Total	%
1	0	0	0	0	1	1	1	1	1	1	1	0	1	1	1	9	66.67
2	0	0	1	1	1	1	1	1	1	0	1	1	1	1	1	11	80.00
3	0	0	0	1	0	1	1	1	1	1	0	1	1	1	1	9	66.67
4	0	0	0	0	1	1	1	0	1	1	1	1	1	1	1	9	66.67
5	0	0	0	1	0	0	1	1	1	1	1	0	1	1	1	8	60.00
6	0	0	0	1	1	1	1	1	1	0	1	1	1	1	1	10	73.33
7	0	0	0	1	1	1	1	0	1	1	0	1	1	1	1	9	66.67
8	0	0	0	0	1	0	1	1	1	1	1	0	1	1	1	8	60.00
9	0	0	0	0	0	1	0	1	1	1	1	1	1	1	1	8	60.00
10	0	0	0	0	1	1	1	1	1	0	1	1	1	1	1	9	66.67
11	0	0	0	1	1	1	1	1	0	1	1	1	1	1	1	10	73.33
12	0	0	1	1	1	1	0	1	1	1	1	1	1	1	1	11	80.00
13	0	0	0	0	0	1	1	1	0	1	1	1	1	1	1	8	60.00
14	0	0	0	1	0	1	1	0	1	1	1	1	1	1	1	9	66.67
15	0	0	0	0	0	1	1	1	1	1	1	1	1	1	1	9	66.67
16	0	0	1	1	1	0	1	1	1	1	1	1	1	1	1	11	80.00
17	0	0	0	0	1	1	0	1	1	1	1	1	1	1	1	9	66.67
18	0	0	0	1	0	1	1	1	1	1	1	1	1	1	1	10	73.33
19	0	0	0	0	1	1	1	1	1	1	1	1	1	1	1	10	73.33
20	0	0	0	0	0	1	1	1	1	1	1	1	1	1	1	9	66.67
21	0	0	0	0	0	1	1	1	1	1	1	1	1	1	1	9	66.67
22	0	0	0	1	1	1	1	1	1	1	1	1	1	1	1	11	80.00
23	0	0	1	1	1	1	1	1	0	1	0	1	1	1	1	10	73.33
24	0	0	0	0	0	0	1	1	1	1	1	1	1	1	1	8	60.00
25	0	0	0	1	1	0	1	1	1	0	1	1	1	1	1	9	66.67
26	0	0	0	0	0	1	1	1	1	1	1	1	1	1	1	9	66.67
27	0	0	0	0	1	1	0	1	0	1	1	0	1	1	1	7	53.33
28	0	0	0	1	1	1	1	1	1	1	1	1	1	1	1	11	80.00
29	0	0	0	0	0	0	1	0	1	1	1	1	1	1	1	7	53.33
30	0	0	0	0	0	1	0	1	1	1	1	1	1	1	1	8	60.00
31	0	0	0	0	1	1	1	1	1	1	1	1	1	1	1	10	73.33
32	0	0	0	0	0	1	1	1	1	1	1	1	1	1	1	9	66.67

Table 2: Responsive and Unresponsive PER Assays in Experimental Group.

(1=Responsive Assay; 0=Unresponsive Assay)

	Trials																
Bee #	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	Total	%
1	0	0	0	0	0	0	0	0	0	0	0	1	0	1	1	3	20.00
2	0	0	0	0	0	0	0	0	0	0	0	0	1	1	1	3	20.00
3	0	0	0	0	0	0	0	0	0	0	0	1	0	1	0	2	13.33
4	0	0	0	0	0	0	0	0	0	0	1	0	0	1	1	3	20.00
5	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	2	13.33
6	0	0	0	0	0	0	0	0	0	0	1	0	1	0	1	3	20.00
7	0	0	0	0	0	0	0	0	0	0	1	1	1	1	0	4	26.67
8	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	2	13.33
9	0	0	0	0	0	0	0	0	0	0	1	1	1	1	1	5	33.33
10	0	0	0	0	0	0	0	0	0	0	1	1	0	1	1	4	26.67
11	0	0	0	0	0	0	0	0	0	0	1	0	0	1	1	3	20.00
12	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0.00
13	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	2	13.33
14	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	2	13.33
15	0	0	0	0	0	0	0	0	0	0	0	0	1	1	1	3	20.00
16	0	0	0	0	0	0	0	0	0	0	0	1	1	1	0	3	20.00
17	0	0	0	0	0	0	0	0	0	0	1	1	1	0	1	4	26.67
18	0	0	0	0	0	0	0	0	0	0	0	0	1	1	1	3	20.00
19	0	0	0	0	0	0	0	0	0	0	0	1	0	1	0	2	13.33
20	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	1	6.67
21	0	0	0	0	0	0	0	0	0	0	0	0	1	0	1	2	13.33
22	0	0	0	0	0	0	0	0	0	0	0	1	0	1	1	3	20.00
23	0	0	0	0	0	0	0	0	0	0	0	0	1	1	1	3	20.00
24	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	1	6.67
25	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	2	13.33
26	0	0	0	0	0	0	0	0	0	0	0	1	1	0	1	3	20.00
27	0	0	0	0	0	0	0	0	0	0	0	1	0	1	0	2	13.33
28	0	0	0	0	0	0	0	0	0	0	0	1	1	0	0	2	13.33
29	0	0	0	0	0	0	0	0	0	0	0	0	1	1	1	3	20.00
30	0	0	0	0	0	0	0	0	0	0	1	0	1	1	1	4	26.67
31	0	0	0	0	0	0	1	0	0	1	0	1	1	0	0	4	26.67
32	0	0	0	0	0	0	1	0	0	1	1	1	0	0	1	5	33.33

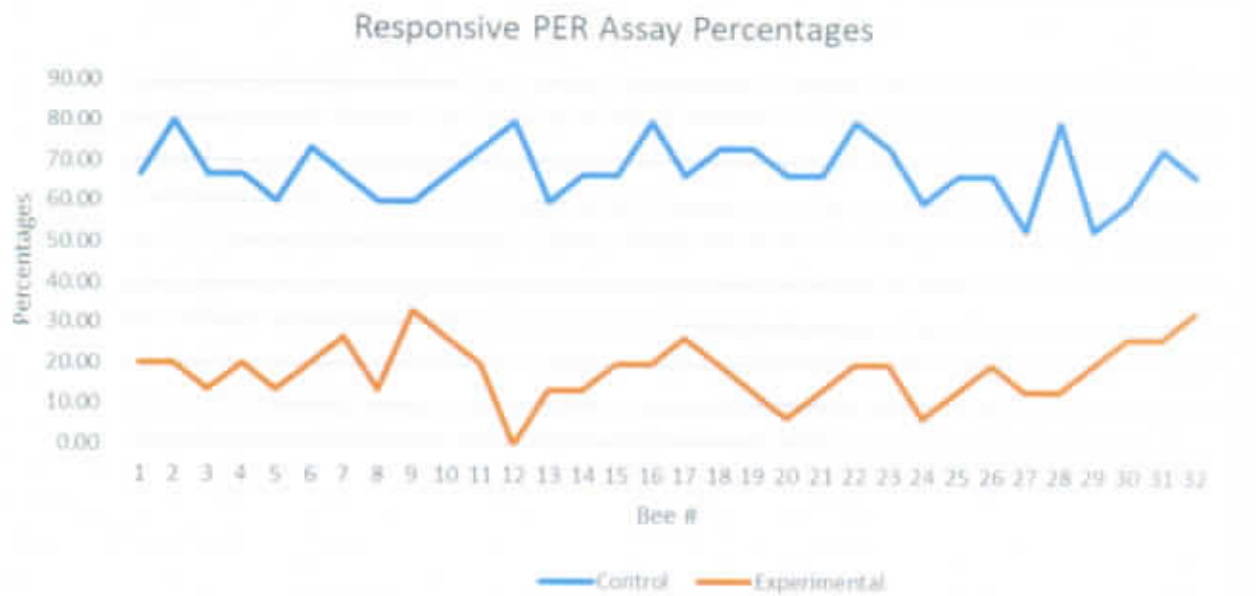

Figure 1: Line Graph of the Control and Experimental PER Assay Percentages.

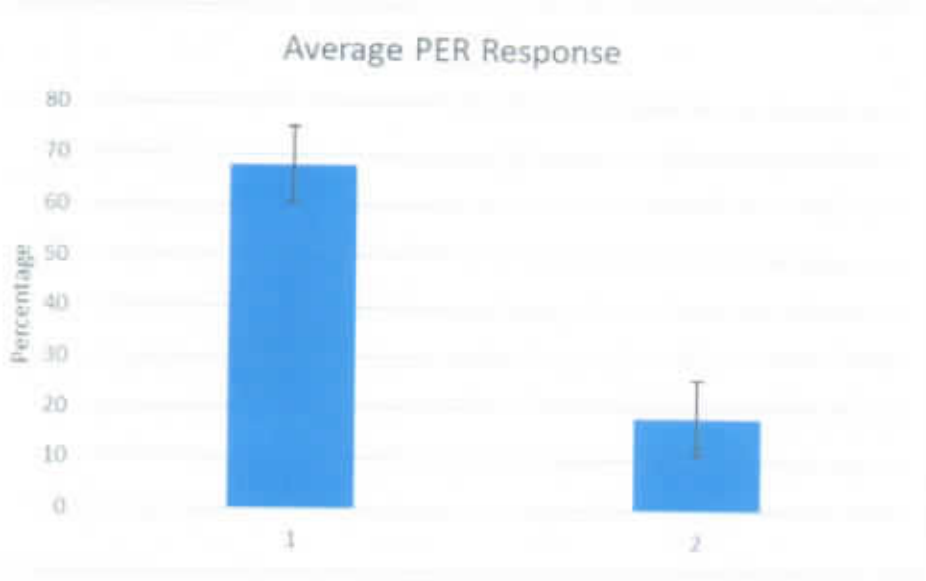

Figure 2: Percentage of Average PER Responses. ***(P-value of 1.00758 E -35).*** Error bars represent standard deviation

DISCUSSIONS AND CONCLUSIONS

The sulfur dioxide (SO_2) created a significant disparity between the learning times of the control and experimental groups, which indicates that it impairs the olfactory system of honeybees. The burning of fossil fuels results in the production of pollutants such as SO_2. This increase in air pollution poses a threat to the population of Western honeybees (*Apis Mellifera*) as it influences their foraging efficiency. Through our trials, we know the extent of how air pollutants reduce their ability to perceive floral volatiles and pheromones. It is imperative to research the effects of potentially harmful pollutants such as SO_2 to honeybees in order to gain knowledge of their dangers and understand how the issue of the honeybee decline can be addressed as they are vital to our ecosystem.

FUTURE RESEARCH & LIMITATIONS

For future studies, we could include the use of electroantennograms to determine if the impairment that pollution causes directly impacts the antennae or the brain, as we are uncertain as to the location of where the olfaction system was affected. It would also be practical to determine if other pollutants that are abundant in our atmosphere impacts olfactory learning and how source type plays a factor. Additionally, seeing as we only focused on the urban, industrial areas of New York, the difference of floral perception among urban, suburban and rural areas could be investigated. Considering our methodology of exposing the honeybees to sulfur dioxide were not precise, replicating this objective with a fixed amount of sulfur dioxide released into the bees' closed space, would produce more exact results.

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