The Effects of Pesticides, Caffeine & Tea Polyphenols on the Visual and Olfactory Learning and Memory of the Honey Bee

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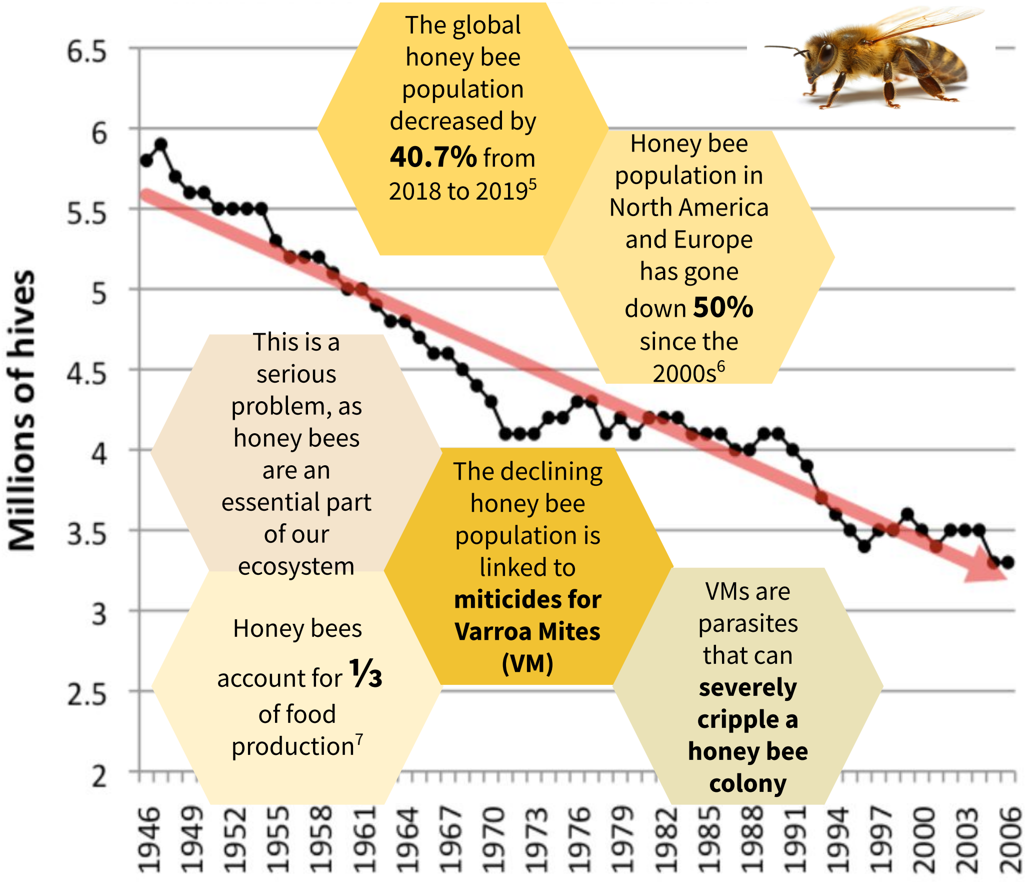
Non RRI Project

The global honey bee population decline is linked to harmful pesticides. To solve this problem, I researched how tea polyphenols and caffeine could repair a honey bee's learning and memory after application of pesticides. I designed my experiment in two parts: Spatial and PER (Proboscis Extension Reflex). The spatial experiment tested how fast a bee could find food in an open setting. The PER experiment used Pavlovian conditioning to teach the bees to recognize both a peppermint scent (olfactory) and the shape of a q-tip (visual). In the end, I discovered that tea polyphenols and caffeine were able to repair the honey bee's learning and memory after application of pesticides and improved it on average by 153%. These findings could save millions of bees if beekeepers feed their bees tea polyphenols and caffeine, repairing their learning and memory skills and combating the negative effects of pesticides.

Science

Project Origin and Research Background

The Decline of the Honey Bee Population



The Significance of My Research

- Harmful miticides cause a decline in learning and memory skills, potentially resulting in Colony Collapse Disorder
- Floral tea polyphenols (TP) have been shown to enhance honey bee olfactory learning and memory
- My research will study if TP can not only enhance but also repair after miticide harm, helping bees find their way home again
- This could save millions of these important pollinators, and perhaps even boost their role in our ecosystem

Research Objectives

- Determine if TP will improve honey bee olfactory learning and memory after impairment via pesticides
- Find if TP will enhance visual learning and memory as well as improve it after impairment via pesticides
- Discover whether bees learn more effectively with sight (visual) or smell (olfactory)

Summary of Literature Review

Chemicals / Details	Oxalic Acid (OA), Formic Acid (FA), and Bayvarol (BA) [1]	cGMP and cAMP [2]	Caffeine (CA) [3]	Floral Tea Polyphenols (TP) [4]
Research Purpose	Determine which of OA, FA, and BA is most effective against VMs and least intrusive in the hive	To test a new paradigm for honey bee spatial memory and learning	To see how CA can boost honey bee learning	To test the effects of TP on honey bee memory retention and olfactory sensitivity
Testing Methods	Mites killed, honey made	Food Search Box (FSB)	Proboscis Extension Reflex (PER), DMST, Y-Maze	PER, EAG Response
Results	OA is the most effective and least intrusive pesticide	FSB is a more efficient paradigm for spatial memory and learning	CA significant for PER, DMST, weak for Y-Maze	TP significantly improved memory and EAG, weakly improved learning

Previous Research Achievements

- Studies have been done on adverse effects of pesticides
- Studies have researched the benefits of CA/TP on olfactory learning and memory
- Similar studies have tested bee's learning and memory in a spatial setting

Research Gaps

- Few research has been conducted on the effects of tea polyphenols on *visual or spatial* learning and memory
- Few have tested if tea polyphenols or caffeine can combat the adverse effects of pesticides

My Research Focus

- Test if TP can enhance visual and spatial learning and memory
- Test if TP and CA can improve learning and memory after pesticide impairment
- Compare the effects of TP/CA on visual vs olfactory learning and memory

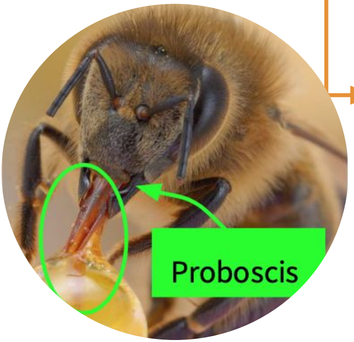
Research Questions and Hypotheses

Research Questions	Research Hypotheses
1 If a pesticide (acaricide thymol, formic acid, or oxalic acid) is applied on a honey bee and memory/learning skills measured, then tea nectar polyphenols are fed to the bee and memory/learning skills measured, will the second trial show improvements from the first, and will it surpass that of the control?	The tea nectar polyphenol will improve honey bee's memory and learning after the application of pesticide. However, it may not be better than that of the control, as TP has been shown to only weakly positively affect bee learning (although strongly affect bee memory)[4].
2 Will bees learn more effectively using their sight or using their smell? Will TP or CA improve more on bee's olfactory or visual learning/memory?	After being fed TP/CA, bees in the olfactory test will outperform those in the visual test because TP/CA have only been shown to enhance olfactory, not visual, learning and memory.
3 Will bees be able to distinguish between different colored Q-Tips?	The bees will be able to tell the difference between blue and yellow colored Q-Tips.

THE EVOLUTION OF THE EXPERIMENT PROCEDURE

Spatial Experiments
- Test if TP/CA can enhance learning or memory spatially
- Test if bees can be trained to search for sucrose in free space
- Explored multiple settings, as shown in next slide

PER Experiments
- Test bee's proboscis extension reflex (PER) to olfactory and visual stimuli after being trained
- Find if TP/CA can repair learning and memory after exposure to pesticides
- Bees harnessed
- Bees adapted to feeding routine easily as opposed to spatial experiments.



Visual

Visual Shape (VS)
- Test if bees can recognize Q-Tip shape
- More bees learned during VS than RO

Visual Color (VC)
- Paired bees with sucrose on blue Q-Tip, none on yellow Q-Tip
- Bees thought any object was food

Olfactory

Crude Olfactory (CO)
- Cotton tape harnesses, paired bees with Clorox Scent and sucrose (SC)
- Bees PER-ed but did not learn.

Basic Olfactory (BO)
- Cardboard tape harnesses, paired bees with peppermint (PM) and SC
- Some bees learned

Refined Olfactory (RO)
- Pure tape harnesses, paired bees with peppermint (PM) and fructose
- Majority of bees learned

Spatial Experimental Procedures

1.0 - Large Training Box (15” × 10” × 7½”)

- Captured and marked bees before placing in box
- Bees panicked, became depressed in box
- Looked for exit instead of food
- Eventually gave up, refused to move
- Spatial test was not as successful as expected

The large training box.

1.1 - Smaller Training Box (6” × 6” × 3½”)

- Drilled ventilation holes on lid
- Froze bee so less panicked
- Still tried to escape, did not search for sucrose initially
- Was feeding on sucrose next day
- This method could work, but takes too long

Smaller training box with bee feeding on sucrose.

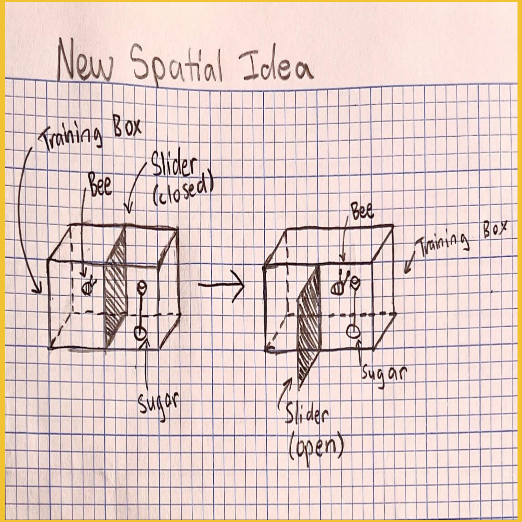
2.0 - Plastic Portion Cups

- Small plastic portion cups with sugared q-tip on top
- Tested if bees could find the q-tip
- Found food but was still panicked, depressed

The plastic portion cup setup.

Key Learning and Future Improvement

- Bees will panic if suddenly placed in a new environment
- Bees will slowly but eventually adapt to new environment
- Next time, spatial box in two parts
- Slider separates bees and sugar
- Open slider during feeding time



PER Experimental Procedures

FINDING THE BEEKEEPER

- Harder than expected
- Wendy from The Honey Ladies → Did not work out
- Bryan → recommendation to email president of Santa Clara Valley Beekeepers Guild.
- Request for help was posted on guild's website
- Anna taught me how to safely handle her bees

COLLECTING THE BEES

- Shook bees into large box and sprayed water on them
- Wings were immobilized
- Scooped up and placed the bees in plastic boxes with ventilation

Olfactory Procedure

Repeat over several learning/memory cycles

Preparation
- Collect bees and freeze them until immobilized
- Strap frozen bees into tape harnesses
- Feed bees TP/CA or expose to FA/AT/OA for 2 hours

Learning Test
- Give SC while exposed to PM for 5s. Take away SC, PM for 5s. Repeat 3x
- Expose to PM (without SC) and see if bee PERs

Repeat until bee learns or is satiated

Memory Test
- After 24 h, retest by exposing to PM without SC

Visual Procedure

Repeat over several learning/memory cycles

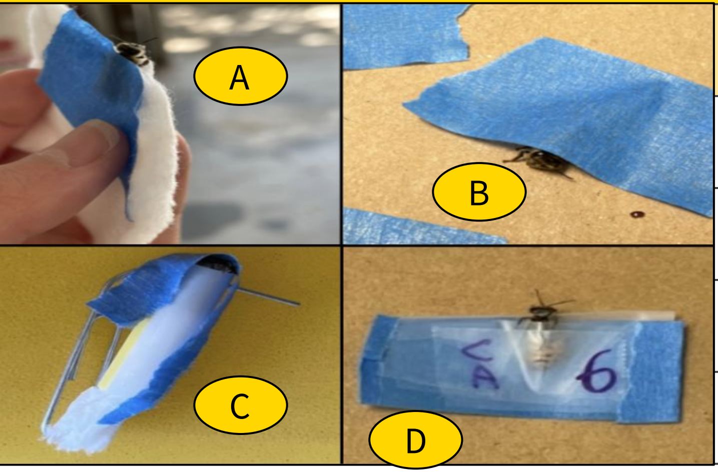
Preparation
- Collect bees and freeze them until immobilized
- Strap frozen bees into tape harnesses
- Feed bees TP/CA or expose to FA/AT/OA for 2 hours

Learning Test
- Feed the bees White/Blue/Yellow Q-tip with SC
- Feed the bees White/Blue/Yellow Q-tip without SC

Repeat until bee learns or is satiated

Memory Test
- After 24 h, retest by exposing to PM without SC

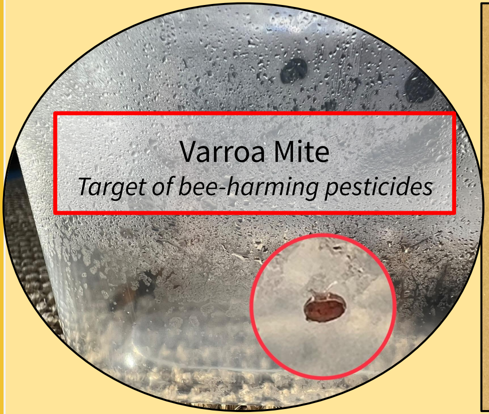
PER Experimental Procedures



Bee Harness Method	Security	Release Difficulty
A. Taping bees to cotton swabs	Very secure	Very hard
B. Taping bees to cardboard	Not secure	Very easy
C. Securing bees in plastic straws	Not secure	Very easy
D. All-tape harness	**Secure**	**Easy**

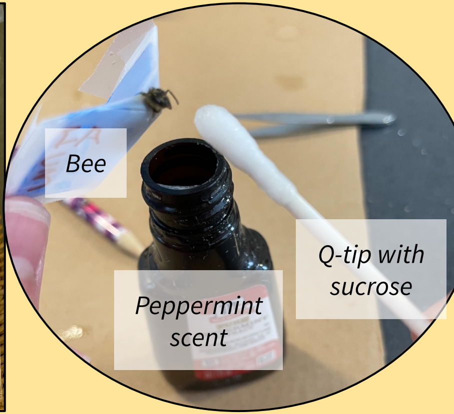
Immobilizing Bees

- Bees had to be immobilized to be strapped into harnesses
- Bees were placed in the refrigerator (~37 F) for 1 - 2 hours
- They woke up after 2 minutes of removal



Container of bees right after removal from fridge


Mass Production of harness method 4



Testing with harness method 4

Results

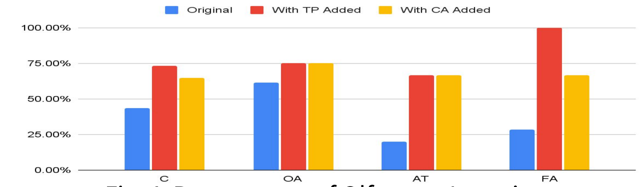


Fig. A. Percentages of Olfactory Learning. TP/CA improved pesticide impairment and made it better than the control. 104 bees were tested.

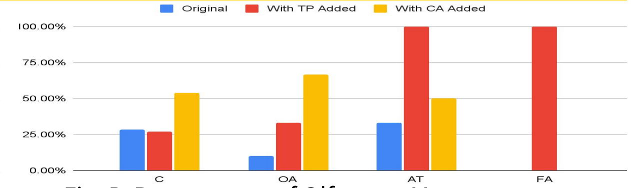


Fig. B. Percentages of Olfactory Memory. TP/CA improved pesticide impairment and made it better than the control. 59 bees were tested.

Fig. C. Percentages of Visual Learning. Both TP and CA improved honey bee learning. TP enhanced the learning of bees exposed to FA more than CA did. 71 bees were tested.

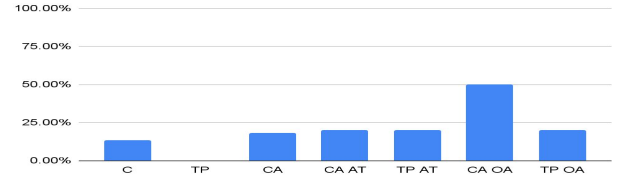


Fig. D. Percentages of Color Learning. Most bees did not respond to color. Adding pesticides or enhancers did not improve color learning. 62 bees were used.

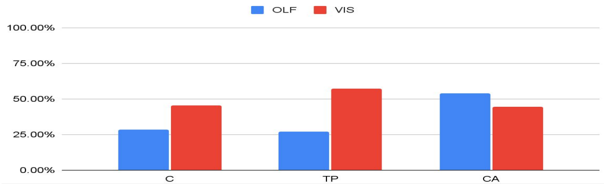


Fig. E. Olfactory vs. Visual (with Color) memory. Visual outperformed olfactory for control and TP. For olfactory 31 bees were used, and for visual 38 bees were used.

Fig. F. Percentages of Visual Memory. FA's unexpectedly high result may have been from a lack of data (only four FA bees were tested for memory). 53 bees were tested in total.

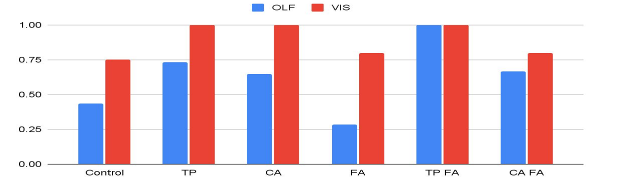


Fig. G. Olfactory vs. Visual (without Color) learning. Visual outperformed olfactory for everything except TP FA. For olfactory 62 bees were used, and for visual 71 bees were used.

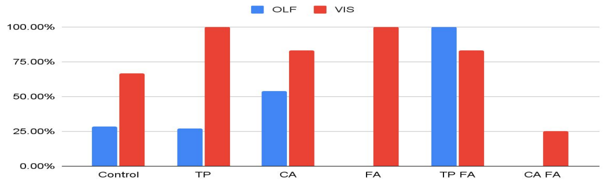


Fig. H. Olfactory vs. Visual (without Color) memory. Visual outperformed olfactory for everything. For olfactory 36 bees were used, and for visual 53 bees were used.

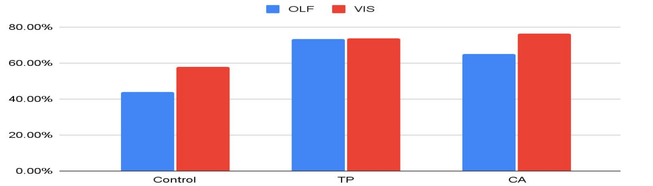


Fig. I. Olfactory vs. Visual (with Color) learning. Visual outperformed olfactory for control, TP, and CA. For olfactory 51 bees were used, and for visual 55 bees were used.

Discussion, Analysis, and Chi-Squared Testing

STATISTICAL ANALYSIS

```cpp
#include <bits/stdc++.h>
#include <algorithm>
using namespace std;

int main() {
  int n, m, tot = 0;
  long double chi = 0;
  cin >> n >> m;
  long double table[n+1][m+1];
  for(int i = 0; i < n; i++){
    for(int j = 0; j < m; j++){
      cin >> table[i][j];
      tot += table[i][j];
    }
    table[i][m] = tot;
    tot = 0;
  }
  for(int i = 0; i <= m; i++){
    for(int j = 0; j < n; j++){
      tot += table[j][i];
    }
    table[n][i] = tot;
    tot = 0;
  }
  long double expected[n][m];
  for(int i = 0; i < n; i++){
    for(int j = 0; j < m; j++){
      expected[i][j] = (table[i][m]/table[n][m])*table[n][j];
      if(expected[i][j] < 5){
        cout << "Not valid for chi-squared testing!";
        return 0;
      }
    }
  }
  for(int i = 0; i < n; i++){
    for(int j = 0; j < m; j++){
      chi = chi + pow(table[i][j]-expected[i][j], 2)/expected[i][j];
    }
  }
  cout << chi;
  return 0;
}
```

χ^2 Contingency Test - OLF_LRN

CONTROL	CHANGE	CHI	DF	RESULT (P<0.05)	RESULT (P<0.1)	RESULT (P<0.2)
C	TP	2.7826	1	Accepted	Rejected	Rejected
C	CA	1.62563	1	Accepted	Accepted	Rejected
C	OA	0.908906	1	Accepted	Accepted	Accepted
C	FA	0.471018	1	Accepted	Accepted	Accepted
C	AT	1.99839	1	Accepted	Accepted	Rejected
TP	TP OA	0.00452381	1	Accepted	Accepted	Accepted
TP	TP FA	0.355556	1	Accepted	Accepted	Accepted
TP	TP AT	0.0553846	1	Accepted	Accepted	Accepted
CA	CA OA	0.15	1	Accepted	Accepted	Accepted
CA	CA FA	0.00319444	1	Accepted	Accepted	Accepted
CA	CA AT	0.00319444	1	Accepted	Accepted	Accepted

χ^2 Data Table

	Learn	Not Learn	Total
C	7	9	16
TP	11	4	15
CA	13	7	20
OA	8	5	13
AT	3	12	15
FA	2	5	7
CA AT	2	1	3
TP AT	2	1	3
CA FA	2	1	3
TP FA	1	0	1
CA OA	3	1	4
TP OA	3	1	4
Total	57	47	104

The olfactory results of my chi-squared testing

DISCUSSION OF DATA ANALYSIS

- TP significantly better than control group for olfactory learning (P<0.1)
- CA significantly better than control group for olfactory learning (P<0.2)
- AT significantly worse than control group for olfactory learning (P<0.2)
- TP, CA improved over pesticide-exposed groups for olfactory memory
- OA, FA worse than control group for olfactory memory
- TP, CA improved over control and FA-exposed groups for shape learning
- TP but not CA improved control but not FA for shape memory
- CA but not TP improved control for color learning
- Most bees did not respond to a difference in color during training
- Visual/color was better than olfactory learning and memory

EVALUATION OF HYPOTHESES

- Hypothesis #1 was **correct** as TP and CA improved learning and memory after impairment from pesticides
- Hypothesis #2 was **correct** as for learning, both TP and CA improved olfactory *(67.61% and 48.57%)* more than visual (*33.33% and 33.33%*). For Memory, CA improved Olfactory (*88.48%*) more than visual (*25%*), TP improved Olfactory (*-4.55%*) less than visual (*50%*)
- Hypothesis #3 was **incorrect** as most bees could not tell the difference; they responded to all stimuli
  - Probably because the bees recognized shape faster than color

Note: I attempted to do a statistical analysis to test my hypotheses. I learned about the chi-squared test from Khan Academy and tried to apply it to my research. Because the sample size might not have been large enough, the test results were insignificant for p < 0.05. I plan to test more bees in the future to see if it would improve statistically.

Conclusion

- This research targeted TP and CA improving bees' memory and learning after impairment from pesticides
- It also looked at how bees recognized shape and color
- Olfactory: TP improved control learning by 67.61%, memory by -4.55%, OA learning by 21.87%, memory by 233.30%, AT learning by 233.35%, memory by 200%, FA learning by 250%
- Olfactory: CA improved control learning by 48.57%, control memory by 88.48%, OA learning by 21.87%, OA memory by 566.70%, AT learning by 233.35%, AT memory by 50.01%, FA learning by 133.36%
- Because TP and CA are effective at improving bees' memory and learning after impairment from pesticides, beekeepers could use TP or CA enhanced sucrose, allowing them to fight the adverse effects of pesticides
- Visual performed better than olfactory - on average, outperformed learning by 26.28%, and memory by 47%
- Bees did not identify color well - control learning was only 13.33%, and control memory was 0%
- I designed a new and easier method to harness bees (see slide 8)
- I plan to do future work in the following directions:
    - Continue to develop statistical analysis for this research by increasing the sample size
    - Using a pump to puff scent at bees rather than holding the bottle underneath them
    - Using my new spatial setting (see slide 7) to improve the spatial experiments

References

[1]Mahmood et al. “Control of Varroa destructor Using Oxalic Acid, Formic Acid and Bayvarol Strip in Apis mellifera (Hymenoptera: Apidae) Colonies” ResearchGate, November 2012.
[2]Tsvetkov et al. “A new protocol for measuring spatial learning and memory in the honey bee Apis mellifera: effects of behavioural state and cGMP” ResearchGate, July 2018.
[3]Si et al. “Effects of caffeine on olfactory and visual learning in the honey bee (Apis mellifera)” ResearchGate, January 2006.
[4]Gon et al. “Floral tea polyphenols can improve honey bee memory retention and olfactory sensitivity” Journal of Insect Physiology, January 2021
[5]Jacobo, Julia. “Nearly 40% Decline in Honey Bee Population Last Winter ‘Unsustainable,’ Experts Say.” *ABC News*, ABC News Network, 9 July 2019
[6]Mooney, Chris. “Bumblebees Are Dying across North America and Europe as the Climate Warms, Scientists Say.” *The Washington Post*, WP Company, 6 Feb. 2020
[7]“US Pollinator Information.” *USDA REE*, United States Department of Agriculture