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The response of Male Bumblebees to a Putative Queen Pheromone

by

Xavier K. Haemmerle

**Capstone submitted in partial fulfillment of
the requirements for graduation with**

UNIVERSITY HONORS

with a major in

**Biology
in the Department of Science**

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Logan, UT**

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Abstract:

Queen pheromones are chemical signals produced by the dominant reproductive female in many species of eusocial insects. These pheromones are vital for maintaining a reproductive division of labor. Two evolutionary scenarios may describe the origin of queen pheromones. Sensory exploitation describes a scenario where the pheromone is produced to take advantage of a preexisting sensory bias in a population. An alternative scenario is that the recipient of the pheromone has an adapted response to a preexisting chemical signal. There is a growing body of evidence that cuticular hydrocarbons that act as queen pheromones are co-opted from ancient fertility signals that existed in solitary ancestral species of modern social Hymenoptera. In many species of Hymenoptera, similar cuticular hydrocarbons have also been identified as sex pheromones. I tested the hypothesis that a putative queen pheromone identified in *Bombus impatiens* originated from a sex pheromone present in an ancestral species with an experiment designed to identify primer and releaser effects of queen pheromones on males. Although behavioral (releaser) results were inconclusive, future analyses of the collected samples may reveal neural or reproductive physiological (primer) responses to the pheromone. This experiment will serve as a foundation from which to design additional studies concerning the evolution of chemical social signals.

Acknowledgments:

I would like to express my gratitude to Dr. Karen Kapheim, my mentor, and Mallory Hagadorn, a doctoral candidate in the Kapheim lab who both helped me greatly in achieving the goals of my project. Their guidance and encouragement provided me with a supportive environment to develop my skills as a researcher. Their advice was vital for the development of my hypothesis and experimental design. I would like to thank James Herndon, who tutored me in the process of dissecting bees and understanding the male bee anatomy. I am grateful to Anna Figgins, an undergraduate student who I had the privilege of working alongside while she completed her project on the role of small non-coding RNA in bumblebee reproduction. We worked together to maintain the bee colonies used in both our projects. Finally, I would like to thank Abby Tucker who provided support for both Anna and me as we completed our separate projects. I am greatly appreciative of her time investment while also being a full-time student.

Final Product: 2311 words

Introduction:

A hallmark of eusociality is a reproductive division of labor within a colony. In insects, chemical communication between individuals is vital for maintaining this social structure. In eusocial Hymenoptera, a class of pheromones called queen pheromones are thought to play a vital role in maintaining a reproductive division of labor across multiple species that have independently evolved eusociality (Holman, 2010). These queen pheromones signal the presence of a queen and prevent other females in a colony from reproducing (Amsalem, et al, 2015) (Oi, Millar, van Zweden , & Wenseleers) (Oliveira, et al., 2015).

Two predominant evolutionary scenarios can explain the origin of queen pheromones in modern social Hymenoptera. One scenario is that the pheromone arose from sensory exploitation (Princen, et al., 2019). Pheromones that arise through sensory exploitation allow the sender to take advantage of preexisting sensory biases (Ng, et al., 2014). A notable example of this is the well-studied queen mandibular pheromone produced by honeybee queens (Kaminski, et al., 1990). The honey bee queen mandibular pheromone not only induces sterility in honeybee workers but also the workers and queens of bumblebees (Princen, et al., 2019). The components of the queen mandibular pheromone have also been found to have an anti-ovarian effect on insects outside of the order Hymenoptera, as demonstrated with fruit flies (Calang, et al., 2018). This indicates that the pheromone acts on a highly conserved physiological pathway in insects that existed long before the pheromone (Camiletti, et al., 2013). A queen pheromone that has evolved through sensory exploitation may have allowed the dominant female in primitively social bees to take advantage of highly conserved genetic pathways and keep subordinate females from reproducing.

An alternative scenario is that the recipient of the pheromone may have adapted to respond to a signal that was already being produced by conspecifics (Chapuisat, 2014). A queen pheromone may be co-opted from fertility signals that reproductive females produced for unrelated purposes to inducing worker sterility or as a byproduct of physiological functions such as ovary activation (Oi, et al., 2015). This signal would not exploit other individuals but would act as an honest indication of the dominant female's reproductive status (Kocher & Grozinger, 2011). Subordinate females that have adapted to optimally respond to this cue would receive inclusive fitness benefits through rearing their sisters (Kocher & Grozinger, 2011).

There is growing evidence that in a variety of social insects, cuticular hydrocarbons act as queen pheromones. Structurally simple hydrocarbons including linear alkanes, alkenes, esters, and fatty acids have been found to act as queen pheromones across several species of Hymenoptera that have independently evolved eusociality (Van Oystaeyen, et al., 2014). In two species of eusocial Vespine wasps, long-chain linear alkanes act as queen pheromones (Oi, et al., n.d.). Across multiple species of ants, cuticular hydrocarbons have been found to play a vital role in maintaining worker sterility (Van Oystaeyen, et al., 2014). Notably, the cuticular hydrocarbon 3-methylhentriacontane was found to act as a primer pheromone to induce worker sterility in the black garden ant, *Lasius niger* (Holman, et al., 2010). Unlike the queen pheromone of honey bees, these queen pheromones likely originated from ancient fertility signals that existed in the solitary ancestors of modern social bees.

If queen pheromones in some social Hymenoptera species originally functioned as fertility-linked compounds, the question arises of what function they originally served. One major hypothesis is that in solitary ancestral species these compounds acted as sex pheromones (Oi, et al., 2015). There are multiple instances of cuticular compounds structurally similar to

queen pheromones acting as sex pheromones in both social and solitary Hymenoptera (Oi, et al., 2015). It has been experimentally demonstrated that extracts of the epicuticular waxes of young female alfalfa leafcutter bees, *Megachile rotundata*, induce mating behavior in males (Paulmier, et al., 1999). In the solitary bee, *Colletes cunicularius* a blend of cuticular hydrocarbons, including straight-chain alkanes and alkenes, acted as a sex pheromone (Mant, et al., 2005). Because sex pheromones are highly susceptible to diversifying selection, if queen pheromones are co-opted from sex pheromones it could explain the diversity observed across several species (Oliveira, et al., 2015).

In previous research, the straight alkane n-Pentacosane (C₂₅) has been identified as a putative queen pheromone in two species of bumblebee, *Bombus terrestris* and *B. impatiens* (Holman, 2014, Holman et al., 2016). This compound has been found to cause worker sterility in *B. terrestris* and may negatively affect worker reproduction in *B. impatiens* (Holman, et al., 2016). Based on the aforementioned discussion, it is hypothesized that:

H1: n-Pentacosane as a queen pheromone in bumblebees is derived from a sex pheromone that existed in a solitary ancestral species.

The experimental design I used tested for both a primer and releaser effect. Pheromones classified as having a releaser effect invoke an immediate change in the behavior of the recipient. Pheromones classified as primers cause long term physiological changes in the recipient through prolonged exposure to the pheromone. The classification of a pheromone as a primer is based on the effect of the pheromone on the recipient. The queen pheromone produced by honey bees acts as a primer on workers because it causes physiological changes in the workers that impact their reproductive capabilities. For drones, however, the pheromone may be considered a releaser

pheromone. The pheromone acts as a male attractant and causes an immediate change in the behavior of the males (Mercer, & Jarriault, 2012).

To test the effects of n-Pentacosane as either a primer or releaser pheromone for males, *B. impatiens* drones were reared as adults without previous exposure to any of the pheromones produced by a queen. Males were segregated into a treatment and control group. Males in the treatment group were exposed daily to the putative pheromone, C25. The behavior of both groups in the presence of the pheromone was assayed. Comparisons between the two groups should yield some insight into the effects of the pheromone. If n-Pentacosane acts as a sex pheromone with a releaser effect then males of both the treatment and control group should in a behavioral assay show a strong preference for the compound over a control. If n-Pentacosane acts as a sex pheromone with a primer effect then males of the treatment group should show a greater preference for the pheromone than the control group.

Methods:

Two queenright *B. impatiens* colonies were obtained from Koppert Biological Systems. The colonies were kept in the cages provided by the company. The cages were modified to have access to the colonies. The original plastic mesh lid was removed and replaced with a Plexiglas lid that included a simple hatch to access the colonies. These were late-stage colonies that were producing males. The colonies came with an adequate supply of sugar water. The colonies were given pollen ad libitum in the form of a pollen dough made with sugar water. The pollen was obtained from Better Bee. The colonies were kept at 27 degrees Celsius and in a humidity range

of 52%-62% rh. A lighting cycle of 8 light 16 dark was used (Aupinel, 1994). Any work done with the bees was done under red light.

To obtain males that had no prior exposure to the pheromones produced by the queen, clusters of pupal cells were excised from the queenright colonies and placed in separate cages. Each cage had an immediate supply of sugar water ready for the bees as they emerged. The cages were stored in the same room as the main colonies and were kept on a Hydrofarm Seedling Heat Mat that made up for the lost heat that would have been provided by other members of the colony. The pupal cell cages were checked daily for newly emerged bees. Newly emerged females were placed in their colonies of origin. Males were placed individually in cages.

Figure 1

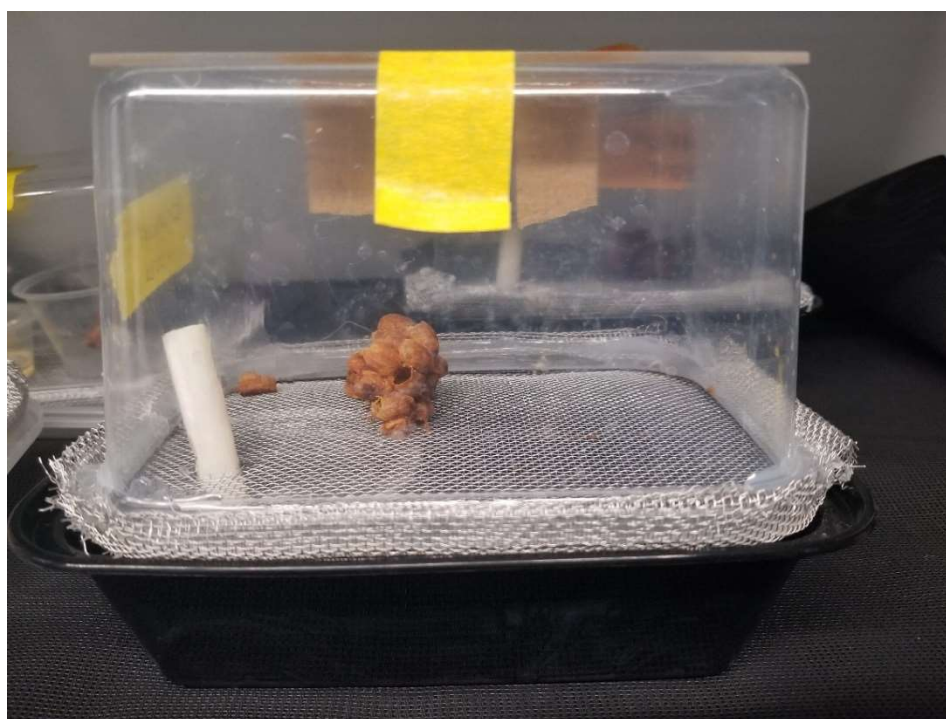


Fig. 1. Cage setup used for pupal cell clusters. Included in this picture is a cluster of pupal cells pulled from a queenright colony. The white wick supplies newly emerged bees with sugar water from a cup bellow the mesh floor.

Of the newly emerged males, half were assigned to the treatment group at random. The remaining males were used as the control group. Males in the treatment group received every 24 hours 2000 ng of C25 (286391, Sigma-Aldrich) dissolved in 5 ul of Hexane (ACS grade, H292-500, Fisher). Males in the control group received only 5 ul of hexane on the same dose schedule as the treatment group. The hexane solution for both groups was pipetted onto a cotton ball placed directly under the mesh floor of the bee's cage. The treatment period lasted for 8 days based on the timing of reproductive maturation in male (Herndon, 2019). The treatment began on the day that the male was removed from the pupal cell cage.

Figure 2

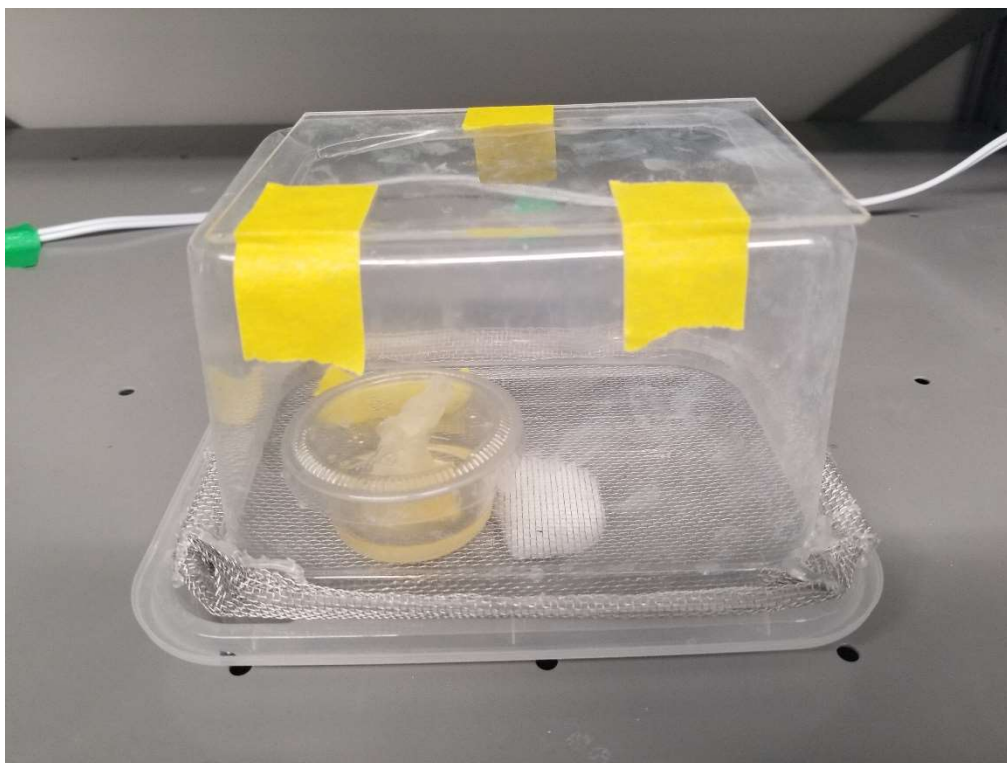


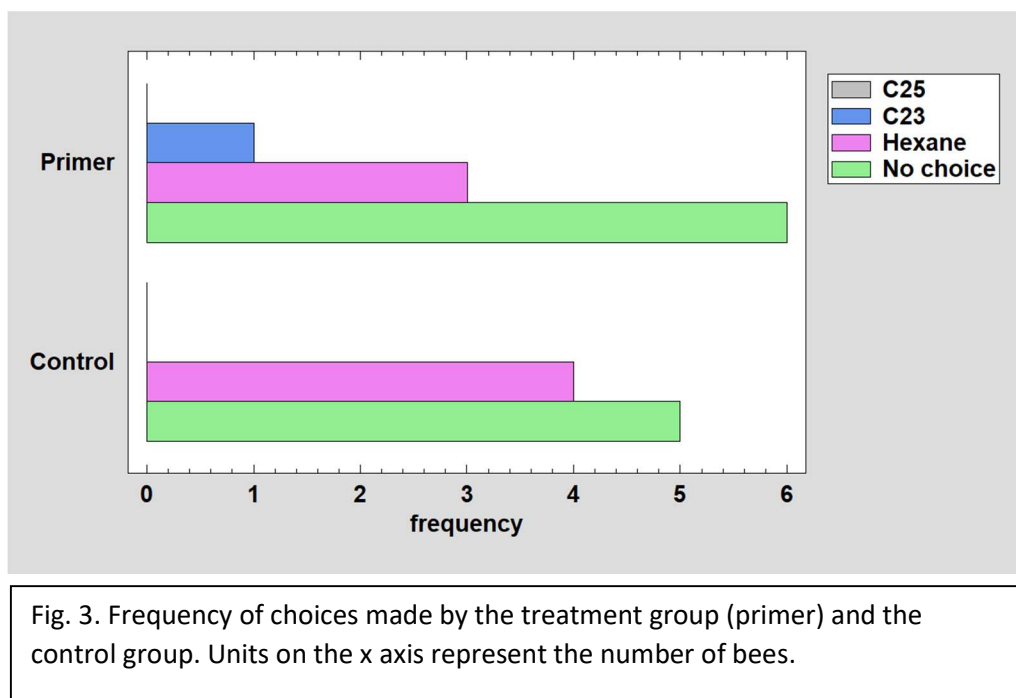
Fig. 2. Cage used for treatment of individual males. Includes supply of sugar water. Cotton ball bellow cage mesh was the site of daily pheromone application. The cotton ball was changed daily.

After 8 days of treatment, the bees from both groups were tested in a behavioral assay. The assay apparatus as pictured allowed the bees to choose between three odors by crawling down the tubes to the cups containing the odor source. In this assay, the bees were given a choice between C25, C23 (263850, Sigma-Aldrich), and Hexane. The C23 was used along with hexane as a control due to it being a straight hydrocarbon similar to C25 that is also found on the cuticle of the queen bee (Amsalem, et al., 2015). For this assay, the odor source was 2000 ng of C25 or C23 dissolved in 5 ul of hexane, and 5 ul of hexane for the hexane control (Amsalem, et al., 2015, Holman, 2014). The bees from both groups were given 30 min to make a choice. The assay was performed under red light.

Results:

The results of my assay yielded no insight on the effect of C25 on male bumblebees as either a primer or releaser. There was an apparent trend for the bees to choose hexane over C23 and C25. However, due to a small sample size caused by extenuating factors that occurred throughout this experiment I cannot form any conclusions regarding my hypothesis based on these results.

Figure 3



Discussion:

Due to the small sample size achieved through the rearing methods utilized in this project I cannot make any conclusions regarding the hypothesis that C25 acts as a sex pheromone with a releaser effect.

A major limiting factor in this experiment is due to the difficulty in rearing males outside of queenright colonies. This method of excising large clusters of pupal cells from queenright colonies proved to be a reliable source of males. However, this method was used late into the project. Despite the success of this method, I was unable to obtain an adequate sample size due to time constraints. Although this rearing method was the most successful for obtaining newly

emerged bees, these males may not have been as naïve as is desired. The males spend most of their development period in their natal colony exposed to the queen's pheromones before they were removed from the colony. This period of development may be more important for the effects of queen pheromones, especially if there is a primer effect (Princen, et al., 2019).

Another factor affecting the success of this project may have been the design of the assay apparatus. This design would be effective with a more volatile odor source. C25 is a waxy substance that would act as a contact sex pheromone. The bees may not have been able to detect the presence of the odor in the assay apparatus. It may be more appropriate to test the effectiveness of these compounds as a male attractant in an olfactometer. By utilizing an olfactometer that allows for laminar like airflow, the test subject would be able to detect the presence and direction of an odor source that may not be highly volatile (Paulmier, et al., 1999).

It is also possible that a single component of a complex pheromone is not adequate to release the attraction response. Most sex pheromones and queen pheromones act as a blend of substances produced by the sender. Sex pheromones produced by virgin bees act as an attractant to males. After mating, the females will lose their attractiveness to males. The queen pheromone produced by the mated queen will then inhibit the reproduction of workers. The main constituents of these pheromones may be similar or the same but varying relative amounts of substances acting synergistically may impact the effectiveness of the pheromone, especially as a sex pheromone (Oi, et al., 2015) (Oliveira, et al., 2015). If this is the case, then males may not respond to a single compound of the pheromone in isolation, even if it is part of the sex pheromone component.

This project is ongoing and future work will focus on the possibility that the pheromone acts as a primer in males. The brains and reproductive organs from the bees used in this

experimented have been preserved. Comparisons between the treatment and control in the development of structures in either the brain or the reproductive system could indicate a possible primer effect induced in males by this pheromone.

Conclusion:

While this study does not offer a conclusive answerer to the questions put forth in this project, it does offer some insight into the effectiveness of the methods uses in the experiment. The evolutionary origin of queen pheromones remains an active area of research that merits further study. Identifying the origins of this pheromone could help to explain the evolution of eusociality in insects due to the importance of queen pheromones and related signals in maintaining social structure in colonies.

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Reflection:

For my undergraduate capstone research project, I chose to design and complete a research project. I wanted to create a project that would test my intellectual independence as well as my ability to think creatively and solve unique problems. Through discussions with my peers and reviewing literature related to my area of study, I identified a substantial knowledge gap in an area that interested me. I began my work on this project with a clear work plan and end goal. Though I was not able to complete the project as intended, with the support of my mentor and peers I was able to work through the problems I encountered through my experiment. The skills I developed through this experience have made this project an ideal way to transition into my intended career.

The topic I chose helped solidify my interest in a specific area of study and helped me gain experience that will help in my career. Though the focus of the project was in a niche area of study, completing this project required me to pull knowledge from multiple fields that I have taken courses in through my time at Utah State University. These areas include animal behavior, evolutionary biology, genetics, biochemistry, and statistics. A capstone project should represent the culmination of a student's knowledge and experience. My project allowed me to demonstrate an ability to practically apply knowledge from my whole undergraduate education. Being able to apply the principals I have learned through my courses required me to demonstrate problem solving and creativity that will be important for my career.

Multiple stages of my project required the same sort of critical thinking that will be expected of me in a research career. The initial development of this project required me to connect ideas from a variety of sources. The hypothesis I tested through my project is based on ideas developed through peer-reviewed research projects that have been published in the past. By

expanding on the work done by these projects I have taken the first steps to make further advancements in related fields of study, especially chemical ecology. Testing the assumptions made by my hypothesis was not a perfectly streamlined process. There were multiple points throughout my experiment when something went wrong, and I was forced to quickly adapt the methods of my project.

There were aspects of my project that I put weeks of labor into that never made it into the final product. In particular, I spent a bulk of my time in this project learning how to rear bees. Despite a significant time investment, for unknown reasons, the rearing method I used failed to produce any bees. Not only did this affect the timeline of my project which caused me to adjust the later portions of my experiment, but it also forced me to develop a new rearing method. While it was at first disheartening to learn that so much of my work had produced no results, I am grateful that I was allowed to experiment with new methods. Ultimately this exposed me to more laboratory procedures and deepened my research experience. I learned more in this project by finding solutions to my failures than I did from the actual end product of the experiment.

Through this experience of working through a variety of problems, I gained a greater knowledge of hypothesis-based testing. Being able to design a repeatable experiment with testable predictions is vital to any field of science. This is an aspect of research I felt very weak in when going into this project. I greatly appreciate how much my mentor was able to help me with forming my research question and experiment. Because of the advice I received from my mentor through the course of designing this project, I feel like I am a far better scientist than I was when I began this project. I know that when I enter into my career I will be more prepared to engage with other scientists.

Though I have not yet begun my post-graduate career through the work I have done with my mentor I feel like I have already begun to engage with the global community of scientists. The topic I chose to study is a very niche topic and while I was doing my supplementary research, I kept seeing the same names come up. However, these researchers represent communities from across the globe. It is difficult to say what the impact of this research would be in any community. Part of the issue is that the topic of queen pheromones in bees other than honeybees is much understudied. Locally, I have found an incredibly supportive community of scientists that I am glad to be a part of. The work I was able to do on my project would not have been possible without the support of other students in the lab run by my mentor. Through engaging with this community, I will have the chance to continue working in research related to pollinators.

The support I received from my mentor and peers has already begun to benefit my career. During this summer I will be employed through the University to work on research related to leafcutter bees. I am not sure what I would have been doing after graduation. It is incredibly reassuring to have a position in a field of research I care about. I am thankful to both my mentor and the other students in her lab. I think that if I continue to work with them, I will be able to gain a better understanding of what I want to do with my career. In return, I am glad to help with the research projects of others.

I am grateful for the opportunities I was provided with through this experience. I know my project suffered from many logistical errors that prevented me from creating the final product I wanted. However, I think the work I did do was more beneficial to me than my intended final product would have been. The development of my skills and character through this project has become a valuable asset that will strengthen my ability to work in a career of research. If I had

any advice to the students who will be starting their capstone next year, that would be to be mentally prepared for failure at some point in their project. Flaws encountered in conducting a research experiment are to be expected. Completing a capstone project should be a challenge and part of that is adapting when something does not work out.

Author Bio:

Xavier Haemmerle is a biologist studying ecology and bio-diversity at Utah State University with a minor in chemistry. Graduating in 2020, he will be continuing his work in the Kapheim lab where he has spent two years of his undergraduate research career. He will be continuing to work in the field of biology with an emphasis on animal behavior and chemical ecology. This will include continued research on queen pheromones while acting as an assistant for research on maternal effects in leafcutter bees.