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The Effects of Climate Change on Plants, Pollinators, and Their Associated Microorganisms

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This dissertation is dedicated to our angel, Holly Vilchez-Russell.

ABSTRACT OF THE DISSERTATION

The Effects of Climate Change on Plants, Pollinators, and Their Associated Microorganisms

by

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Dr. Quinn McFrederick, Chairperson

Floral nectar harbors a community of microbes that affect plant and pollinator fitness. Dynamic and complex plant-pollinator-microbe interactions are likely to be influenced by a rapidly changing climate, as each have their own optimal growth temperatures and phenological responses to environmental triggers, such as temperature. As pollinators forage, the microbes they inoculate into the nectar can alter nectar properties including volume and chemistry. Through a combination of 16S rRNA gene sequencing, quantitative PCR, culturing, manipulative lab and field studies, and large-scale ecological research I attempt to establish the effects of climate change on plants, pollinators, and their associated microbes. In a lab experiment, for Chapter 1, I demonstrated that the common eastern bumble bee, *Bombus impatiens,* prefers nectar inoculated with microbes but incubated at a non-climate change induced

temperature. Next, I used a passive-heating technique (Chapter 2), and an elevational gradient (Chapter 3), to assess the influence increased temperature had on the nectar of *Penstemon heterophyllus*. In Chapter 2, I established that extreme temperature events influence nectar microbes more than small incremental temperature changes. I later found, in Chapter 3 that nectar microbial communities do shift along an elevational (temperature) gradient, but the implications of this shift on pollinators is dynamic and complex. This research reveals that climate change can affect pollinator networks in ways not previously described and leads to better understanding of how to best conserve our plants and pollinators.

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INTRODUCTION

Microbial symbionts, whether facultative or obligatory, are nearly ubiquitous in plants and animals. These symbionts interact with their host on a continuum ranging from mutualistic to parasitic. A major goal of microbial ecology has been to investigate microbial diversity and function in relation to the host. For example, gut symbionts can protect the host from pathogenic infections, as with bumble bees and the parasite *Crithidia bombi* (Koch & Schmid-Hempel, 2011). Also, the volatile organic compounds emitted by the nectar-inhabiting yeast, *Metschnikowia reukaufii,* are attractive to a range of floral visitors including honey bees (Rering et al., 2017) and the aphid parasitoid *Aphidius ervi* (Klaps et al., 2020). This provides protection for the plant host from plant-feeders along with the benefit of attracting pollinators. The role that microbial symbionts play in host interactions and ecosystem processes is a valuable tool which can be used in many ways.

Pollinators, mainly wild and managed bees, provide an essential ecosystem service, however many species are experiencing rapid population declines (Brown & Paxton, 2009). Pollination not only produces a steady food source for many animals, but also facilitates biodiversity (Klein et al., 2007). Pollinators play a role in shaping plant communities, which cascades up to influence diversity of many species (Klein et al., 2007; Ricketts et al., 2004). Humans have harnessed the incredible rate at which bees are able to pollinate by domesticating the honey bee, *Apis mellifera,* and a handful of other species,

which has directly contributed to the decrease in global hunger rates (Tilman et al., 2001). Pollination services contribute roughly $18 billion a year revenue in the United States (Calderone 2012). Although managed bees, such as the honey bee, bumble bee, and alfalfa leaf cutting bee, are credited with the majority of agricultural pollination, wild bees are just as, if not even more, efficient at pollinating crops (Garibaldi et al., 2014). There are many factors that play a role in bee population declines, including habitat loss, climate change, parasite and pathogens, and the use of pesticides (Brown & Paxton 2009; Goulson 2015). Teasing apart the multifaceted interactions within pollination networks, and how each individual player is affected by aforementioned stressors may shed new light on bee health and aid conservation efforts.

Pollination networks include not only the plant and the pollinator, but also their associated microorganisms. Several microbes can survive on flowers and both wild and managed bees (on or within the bee itself or within nest material, Anderson et al., 2013; Figueroa et al., 2019; McFrederick et al., 2016). These close relationships allow the microbes to influence pollination interactions in a variety of complex ways, both positively and negatively. Pathogens of bees persist on floral surfaces, allowing transmission of these deleterious microbes to the pollinator (Durrer & Schmid-Hempel, 1994; Figueroa et al., 2019). However, potentially beneficial microbes such as yeasts (Vannette et al., 2012) and *Lactobacillus* (Vuong et al., 2019) can also be picked up on flowers by

pollinators. Therefore, flowers provide food for bees, but are also hubs for microbial transmission.

Microbes associated with plants and pollinators can play an active role in modifying floral resources and plant cues. Microbes that inhabit plant nectar can modify the nectar chemistry itself. For example, two common nectar inhabiting microbes, *Neokomagataea* (formerly *Gluconobacter*) and *Metschnikowia*, alter nectar sugars and nectar pH very differently (Vannette et al., 2012). *Neokomagataea* acidifies the nectar and metabolizes glucose and sucrose more than *Metschnikowia*, whereas *Metschnikowia* metabolizes fructose more and keeps a more neutral pH in the nectar (Vannette et al., 2012). Honey bees prefer nectar that was inhabited with *Metschnikowia* over nectar with *Neokomagataea* or sterile nectar. These results demonstrate the multifaceted way microbial symbionts influence the pollination network. However, little investigation has been conducted on the effect of abiotic factors on this system.

Warming due to climate change has caused an increase of 0.85 °C in global surface temperatures over the past century (Voosen, 2021). Models based on low CO_2 emission estimate that there will be another 1.5°C rise by the turn of the century (Rogelj et al., 2018). Sixteen of the 17 hottest years in the past 138 years have occurred in the 2000's with 2016 and 2020 being the hottest years on record (Voosen, 2021). This rapid increase in global temperature affects the biology, behavior, ecology, and symbiotic relationships of many plants and pollinators (Hegland et al., 2009; Memmott et al., 2007; Rafferty et al., 2015).

There are many ways temperature changes can indirectly affect plant-pollinator mutualisms.

While these studies of the effects of microbes or climate change on pollination mutualisms have increased our understanding of these separate processes, no studies have combined these fields to predict how climate change will affect the role of microbes in plant-pollinator interactions. Here I attempt to fill this knowledge gap by performing laboratory and field-based experiments that combined the effects of climate change, nectar microbes, and pollination. Altering nectar microbial communities can then affect pollinator preference, pollinator health, and pollination success by altering nectar sugars, secondary metabolites, and volatiles. Understanding this complex tripartite interaction is vital as the pressures of rapid climate change continues to increase.

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CHAPTER 1

Elevated temperature may affect nectar microbes, nectar sugars, and bumble bee foraging preference

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ABSTRACT

The study of plant-pollinator networks is beginning to incorporate the interaction of the microbial communities associated with flowers and their pollinators. Nectar, an important resource for pollinators, is inhabited by microbes such as yeasts and bacteria, which have been shown to influence pollinator preference. Dynamic and complex plant-pollinator-microbe interactions are likely to be influenced by a rapidly changing climate, as each have their own optimal growth temperatures and phenological responses to environmental triggers, such as temperature. To understand how warming due to climate change is influencing nectar microbial communities, we incubated a natural nectar microbial community at different temperatures and assessed the subsequent nectar preference of the common eastern bumble bee, *Bombus impatiens.* Bumble bees preferred nectar inoculated with microbes and incubated at a lower temperature over all other treatments but did not differ in preference of sterile nectar versus inoculated nectar incubated at a climate change predicted temperature. The microbial community in floral nectar is often species-poor, and we were able to isolate and identify only the bacterium *Fructobacillus*. Temperature directly influenced the *Fructobacillus* in nectar with an increase of abundance in the warmer treatment. This increase in abundance altered nectar sugars and lead to significant differences in pollinator preference. These data show that climate change will likely alter plant-pollinator-microbe interactions.

INTRODUCTION

Plants and pollinators provide an iconic example of mutualisms, but nested in this symbiosis is another set of interactions, namely that between flowers, pollinators, and their associated microorganisms. The center of this interaction web is floral nectar. Nectar is a vital part of plant-pollinator interactions, as it is the plant's main mechanism for attracting and rewarding pollinators. Pollinators rely on nectar as a valuable source of energy (Nicolson et al. 2007). However, nectar is more than a sugar-rich resource and surveys of many wild plant species in varied ecological regions have revealed nectar is often inhabited by bacteria (Álvarez-Pérez et al., 2012; Fridman et al., 2012) and fungi (mainly yeast, e.g. Herrera et al. 2009) .

Nectar-inhabiting microorganisms have been found to alter nectar chemistry and influence pollinator behavior (Vannette and Fukami 2016). Nectar, however, can be a harsh environment for microbes as the plant has many ways to combat microbial colonization (Adler, 2000; Herrera et al., 2010). Microbes utilize resources within floral nectaries to change nectar chemistry in many ways. For example, yeasts can ferment plant nectar, creating different floral odors emitted by the plant (Goodrich et al. 2006), as well as change sugar concentrations as they metabolize nectar nutrients (Vannette et al., 2013). Although fungi and bacteria can reduce overall nectar sugars when compared to sterile nectar, bees tend to prefer nectar colonized with microbes (Schaeffer et al. 2017; Vannette, Gauthier, and Fukami 2013). These microscopic changes in

nectar lead to drastic changes in pollinator behavior, however, little is known about how abiotic stressors will affect this interaction.

Rapid increases in global temperature have the potential to disrupt many ecological processes. Warming due to climate change has caused an increase of 0.85 °C in global surface temperatures over the past century (Keohane & Olmstead, 2016). Low CO_2 emission models predict that there will be an estimated 1.5°C rise by the turn of the century (Rogelj et al., 2018). Sixteen of the 17 hottest years in the past 138 years have occurred in the 2000's with 2016 being the hottest year on record so far (Blunden & Arndt, 2017). Most living organisms, including microorganisms, have an optimal living temperature in which they thrive (Ratkowsky et al., 1983; Savage et al., 2004). As temperatures increase, many species interactions will be disrupted (Ogilvie et al. 1997). Although there is ample research on plant phenological and physiological change in the context of climate change, there currently is a lack of information on how climate change will influence plant-pollinator-microbe interactions. Microbial growth is influenced by temperature, often increasing metabolic rate (Ratkowsky *et al.* 1983), which may allow for intensified competition within nectar-inhabiting microbial communities. For example, an increase in temperature may benefit one species of microbe which allows it to outcompete other microbes in that community (Ogilvie et al 1997).

With the looming prospect of rapid temperature increases, understanding the effects of climate change on plant-pollinator interactions is of great

importance. We hypothesize that environmental temperature mediates microbial community structure in nectar, as each microbe develops at their intrinsic optimal growth temperature. We predict that nectar-inhabiting microbial communities will differ between temperature treatments, as well as nectar chemistry and ultimately bumble bee preference. Here we test the effects of two different temperature treatments, one representing baseline temperatures and one representing climate change predicted temperatures for the turn of the century, on the same starting microbial community using synthetic nectar. We determine how temperature-mediated changes in microbial communities alter nectar chemistry and pollinator preference. Our results help tease apart the mechanisms within the plant-pollinator-microbe interactions that will be affected by climate change.

MATERIALS and METHODS

Brassica rapa and nectar extractions

Brassica rapa is a wild mustard introduced to North America from Europe which is pollinated by many invertebrate species. We collected *Brassica rapa* subsp. *oleifera* (Brassicaceae) flowers in the early morning (between 8:00-10:00am) from a meadow in Beaumont, California (33.933670, -117.002738), and brought the flowers back to the lab for nectar extractions. Although the flowers were not bagged, we collected them early in the morning before many pollinators were out foraging. *Brassica rapa* inflorescences have 2-5 flowers open at a time, and we gently removed all unopened floral buds prior to nectar

extraction. To avoid pollen and pollen microbes contaminating the nectar, we used sterile micro-dissecting scissors to carefully remove anthers and pollen from each flower. To extract nectar, we placed 2-5 flowers facing down in a sterile, modified 1.5ml Eppendorf tube and centrifuged the flowers at 7500 x g for 1 minute to remove nectar. To prevent any debris or small invertebrates from falling into the nectar during centrifugation, we modified the 1.5mL Eppendorf tubes by gluing fine mesh halfway up the tube. We used centrifugation as the method to collect nectar as the Brassica flowers are too small to use capillary tubes. Although this method may introduce microbes from the petals or bracts it is unlikely that these microbes would thrive in the artificial nectar as they are phyllosphere bacteria. We sterilized all 1.5mL collection tubes with mesh modifications by UV (254 nm) sterilization in an AirScience UV-Box (Fort Meyers, FL) for 20 minutes prior to use. We pooled nectar extractions for chemical composition analysis and microbial community characterization.

Nectar analysis

To quantify *B. rapa* nectar carbohydrate concentration, we used the Megazyme Sucrose, D-Fructose, D- Glucose Assay Kit to analyze the sucrose, glucose, and fructose concentrations of the nectar. To identify the amino acid composition of the nectar samples, we sent *B. rapa* nectar to Texas A&M University Proteomics department where there is an established free amino acid assay for plant nectar using high-performance liquid chromatography (HPLC, Supplemental Material 1-1). Based on these two analyses of *B. rapa* nectar, we

designed sterile, synthetic nectar as follows: 74g of molecular grade sucrose, 58g glucose and 11g D-fructose in 1L of water (volume x weight), autoclaved, to which was added 8g of Minimum Essential Medium (MEM) sterile Non-Essential Amino Acid Solution (100×) (Sigma-Aldrich M7145).

Choice experiment

We inoculated synthetic nectar with nectar-inhabiting microbial communities from wild *B. rapa* flowers by adding 50 µL of pooled, freshly extracted *B. rapa* nectar (as described above) to synthetic nectar and allowed 24 hours for growth at 25 °C. Then we gently vortexed this single pool of inoculated artificial nectar and added 10 µL to 100 individual 2 mL tubes of synthetic nectar. We then separated these tubes into two treatments of 50 tubes each and incubated each treatment at one of two temperature treatments. The first temperature treatment (27 °C) represents the average spring-time high in Riverside, CA (US Department of Commerce), where the nectar microbes were collected, and the second treatment (32°C) represents a climate change predicted temperature (National Research Council, 2006). According to the National Research Council (2006), high CO_2 emission scenarios predict a 5°C increase in global temperatures by the turn of the century. We incubated treatments for three days, which is the longest that nectar will sit in the nectary of *Brassica* before being depleted or the flower senesces (Masierowska, 2003). For controls, we also incubated 50 tubes of sterile synthetic nectar at each of the temperature treatments. After three days of incubation, we presented 1-5 day old

female worker *Bombus impatiens* with a choice assay to assess feeding preference. We purchased five *B. impatiens* colonies from Koppert Biological Systems (Howell, MI) and maintained these colonies with pollen and 60% sterile sucrose water *ad libitum* in environmentally controlled rooms at University of California Riverside, which were held at 27°C. We assayed 10 bumble bees from each colony for a total of 50 bumble bees. To perform the choice assay, we put one bumble bee in a small foraging chamber (9.5 cm x 10.15cm) that had access to 4 feeders each filled with 1 mL of one of four treatments: (1) synthetic nectar inoculated with microbes and incubated at 27°C; (2) sterile synthetic nectar incubated at 27°C as a control; (3) synthetic nectar inoculated with microbes and incubated at 32°C; and (4) sterile synthetic nectar incubated at 32°C as a control. We point out that the temperature treatments occurred before the choice assay; all 4 nectar treatments were offered to bees at the same temperature as the bees. The bees themselves were not exposed to any temperature treatments but instead held at a constant 27 °C. To assess bumble bee preference, we carefully weighed each feeder before and after a 24-hour foraging period and counted choice as amount in grams of nectar consumed by each bee. Each bee participated in only one choice test and was not returned to the colony after the assay was completed. To be sure that the experimental bees were not acclimated to any of the offered treatments, we did not train the bees to the assay arenas or to the artificial nectar, thus the experimental bees were naive to all four offered treatments in the choice assay. As experimental bees were reared

in their respective colonies, it is possible that they were acclimated to hive microbial contaminants in the sugar water while in the colony. However, the lab colonies we used for these experiments were never exposed to environmental microbes and we regularly changed their sterilized sucrose solution. The shared sucrose source in the colonies would therefore only be exposed to the specialized bumble bee gut microbiota, which is closely related to the honey bee microbiota and is not known to grow in sucrose-only media (Engel et al., 2013). However, previous work done has described the commonly found microbes in commercial bumble bee microcolonies and none of which were sequenced in our nectar treatment (Meesus et al., 2013). Exposure of our experimental bees to nectar-inhabiting microbes before the choice trials is therefore highly unlikely if not impossible.

Extreme heat experiment

We conducted a second choice assay using the same methods as described above but with more extreme temperatures. Using the *Brassica rapa* synthetic nectar recipe, we inoculated the same wild *Brassica* flower microbial community and incubated the nectar for 3 days. In this experiment, we used 32°C and 42°C to incubate the nectar before offering it to 50 bumble bees from 5 different colonies as a choice assay as described above.

Post-assay nectar and microbiota analysis

After the 3-day incubation we divided each volume of the mature microbial communities into two aliquots: one for characterization of the microbial

community and nectar sugars, and one for the bumble bee choice assay. To characterize microbial communities, we centrifuged the aliquoted sample at 4500x g for 5 minutes to pellet out microbes. Once the pellet was formed, we pipetted nectar off for carbohydrate analysis, leaving the pellet for DNA extraction. To measure carbohydrate concentrations on a subset of samples- 10 samples from each treatment, we again used the Sucrose, D-Fructose, D-Glucose Assay Kit (Megazyme, Chicago, IL) to characterize differences in nectar sugars according to temperature treatments.

We extracted DNA from the remaining pellet of all 100 samples using the DNeasy Blood and Tissue Kit (Qiagen, Valencia, CA). To control for possible reagent contaminants, we included N=1 'blank' samples that contained no cells beyond those that may have occurred in the reagents or via possible contamination. We ran these blank samples through all of our library preparation and analysis pipeline. To prepare the samples for extraction, we used a Qiagen tissue lyser to bead-beat samples for 6 min at 30 hz with two sterile 3.2mm chrome-steel beads and roughly 100 µl of 0.1mm glass beads (Biospec, Bartlesville, OK), in 180 µl of buffer ATL from the Qiagen extraction kit. We then added 20 µL of Proteinase K, incubated the samples overnight at 57 °C, and followed the DNeasy standard extraction protocol.

To characterize the microbial communities within nectar, we used dual-index inline barcoding to prepare samples for sequencing on the MiSeq sequencer (Illumina), following the same protocols as detailed in McFrederick

and Rehan (2016). We used primers that included either the forward or reverse Illumina sequencing primer, a unique 8-nt-long barcode, and the forward or reverse genomic oligonucleotide (Kembel et al., 2014). We used the bacterial 16S rRNA sequence primers 799F-mod3 CMGGATTAGATACCCKGG (Hanshew et al., 2013) and 1115R AGGGTTGCGCTCGTTG (Kembel et al., 2014) and the fungal internal transcribed spacer (ITS) primers ITS1F (50 - CTTGGTCATTTAGAGGAAGTAA-30) and ITS4R (50 - TCCTCCGCTTATTGATATGC-30). We performed PCRs using 10 μL of 2× Pfusion High-Fidelity DNA polymerase (New England Biolabs, Ipswich, MA), 10 μL of ultrapure water, 0.5 μL of each 10 μM primer stock, and 4 μL of DNA, with an annealing temperature of 57°C for 30 cycles. We cleaned this product using Ultraclean PCR cleanup kit (MoBio, Carlsbad, CA), to remove unincorporated primers and dNTPs. To complete the Illumina sequencing construct, we used 1 μL of the clean PCR product as a template for a second PCR, using HPLC-purified primers: CAAGCAGAAGACGGCATACGAGATCGGTCTCGGCATTCCTGC and AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACG (Kembel et al., 2014). We then normalized 18 ul of PCR product using SequalPrep Normalization plates (Thermo Fisher Scientific, Waltham, MA). We pooled 5ul of each sample and performed another Ultraclean PCR cleanup on this combined sample. We assessed library quality using a 2100 Bioanalyzer (Agilent, Santa Clara, CA). After quality control, we sequenced the libraries using a MiSeq

sequencer (Illumina) and MiSeq Reagent kit, version 3 (Illumina), with 2 × 300 cycles, at the IIGB Genomics Core, UC Riverside.

Quantification of the microbial community (qPCR)

To determine the absolute abundance of bacterial cells in the artificial nectar samples of both temperature treatments, we used quantitative real-time PCR (qPCR) of the inoculated DNA extractions as above, N=100, however, 30 samples failed to amplify (N=70). Each PCR reaction consisted of 7.5 µL SsoAdvanced master mix (Bio-Rad Laboratories, Los Angeles, CA), 3.6 ml molecular grade water, 0.45 µL forward primer, 0.45 µL reverse primer and 1.0 µl sample or standard DNA. We used the universal bacterial primers for the conserved 16S rRNA region Univ331F as our forward primer (5'-TCCTACGGGAGGCAGCAGT -3') and Univ797R as our reverse primer (5'-GGACTACCAGGGTATCTAATCCTGTT-3') (Nadkarni *et al.* 2002). We used this 16S qPCR primer set instead of 799F-1115R due to its established use in previous microbiome studies (Gandolfi et al. 2015; Hospodsky *et al*. 2015; Hospodsky et al. 2014; Nadkarni *et al.* 2002). The reaction conditions were an initial heating at 95°C for 3 mins, followed by 39 cycles of 95°C for 10 s, 59°C for 30 s on a BioRad C1000 Touch thermal cycler. We compared our samples to a standard curve of $1x10^2$-$1x10^8$ copies of the 16S rRNA gene cloned into a TOPO-TA plasmid (Invitrogen, Carlsbad, CA), with all qPCR efficiencies between 90% and 100% and R^2 above 0.99.

Statistical analysis and microbiome bioinformatics

We used a two-way analysis of variance (ANOVA), Kruskal-Wallis chi-squared test, and TukeyHSD post-hoc pairwise comparison to assess the differences in whether temperature treatment affects nectar sugar concentrations (total sugars as well as individual sugars). We used a linear mixed model with Gaussian error distribution (GLMMs; package lmer) to assess differences in nectar consumption by bumble bees during the choice assay. We used nectar consumption as the response variable, temperature treatment and presence or absence of a microbial community as fixed effects, and colony of origin as random effects. We used package lmerTest to compare coefficients of fixed effects. To test if bacterial cell copies were different between temperature treatments, we used Welch's t-test. We performed all of the above statistical analyses in R 3.4.4. We were unable to amplify fungi from our artificial nectar samples, and therefore do not consider fungi further. We used QIME2-2018.6 (Bolyen et al., 2018) to process the 16S rRNA gene sequence libraries. We trimmed the low-quality ends off the reads with QIIME2's default settings. Next, we binned our sequences into exact sequence variants (ESVs) using DADA2 (Callahan et al., 2016), followed by chimera removal using the default settings of the DADA2 Pipeline. To assign taxonomy to the ESVs we used the QIIME2 q2-feature-classifer (Bokulich et al., 2018) trained to the 799-1115 region of the 16S rRNA gene and conducted local BLASTn searches against the NCBI 16S microbial database (July 2017). We cleaned the data by filtering out ESVs from

the resulting feature table that corresponded to contaminants of reagents as identified in our blanks (Supplemental Table 2). As the artificial nectar in which the floral microbiomes were incubated contained no plant material, we found no plant plastid contamination in our sequencing reads. To generate a phylogenetic tree of our sequences, we used the MAFFT aligner (Katoh & Standley, 2013) and FastTree v2.1.3 (Price et al., 2010). We used rarefaction analysis to determine a standardized coverage of bacterial species diversity to be used in alpha and beta diversity analyses of the DNA extracted from synthetic nectar after incubation period. At 2600 reads per sample we found that the rarefaction curves levelled off, yet most samples could be included in the subsequent analyses. We used this tree and ESV table for alpha diversity analysis and to calculate unweighted UniFrac distance matrices. We used the Shannon Diversity Index and the Kruskal-Wallis test in QIIME2 to analyze alpha diversity. We analyzed differences in beta diversity using Adonis (type II sum of squares) with the vegan package in R (Oksanen et al 2019).

RESULTS

Sugar changes with temperature and microbes

After the three-day incubation period, artificial nectar sugars were significantly reduced when microbes were present (Kruskal-Wallis χ^2= 13.391; N = 50, p<0.0001; Figure 1-1). As microbes consume nectar resources, overall nectar sugars decreased in the inoculated treatments compared to sterile nectar.

Temperature treatment and presence of microbes influenced individual sugar concentrations. There was no significant difference in sucrose levels between any of the four treatments (Kruskal-Wallis χ^2= 2.7792, df = 3, p-value = 0.4269; Fig 2). There was an interaction effect of temperature and treatment on fructose levels ($F_{1,36}$=38.8, p<0.0001; Figure 1-2). There was more fructose in the 27°C compared to 32°C (Tukey HSD, p<0.0001), and significantly more in the sterile nectar compared to nectar with microbes (Tukey HSD, p<0.0001). There was also an interaction effect between temperature and treatment on glucose levels ($F_{1,36}$=10.505, p=0.002; Fig 1-2) with significantly more glucose in 32°C sterile treatment (Tukey HSD, p=0.003), 27°C microbe-inoculated treatment (Tukey HSD, p=0.001), and 32°C microbe-inoculated treatment (Tukey HSD, p<0.0001).

Bumble bee preference

When presented with the four nectar treatment choices, *B. impatiens* consumed significantly more synthetic nectar inoculated with microbes (GLMM; t=6.854, *df*=207, p< 0.0001, Figure 1-3) and incubated at 27 °C – the representative ambient temperature (GLMM; t=-4.190, *df*=207, p< 0.0001, Fig 3), than all other nectar choices. Bumble bees preferred this treatment 1.5 times more than synthetic nectar without microbes, as well as nectar with microbes but incubated at elevated temperature. Similarly, in our “extreme heat” study, bumble bees preferred nectar that had been inoculated with a microbiome and incubated at the lowest of the two temperature treatments (Supplemental Material 1-2).

Microbial community

There was a total of 397,885 quality-filtered reads with an average of 5604 reads per sample (N = 71) that clustered into 205 filtered exact sequence variants (ESVs) for bacterial sequencing. Fungal sequencing showed no fungal (or yeast) growth in our synthetic nectar. We found that there was no significant difference in alpha diversity, using the Shannon Diversity Index, between temperature treatments (Kruskal-Wallis χ^2= 1.3532, *P* = 0.244. Non-metric Multidimensional Scaling (NMDS) analysis on the Generalized UniFrac distance matrix (Figure 1-4) showed that there was no obvious clustering by treatment. We analyzed the Generalized UniFrac distance matrix of our samples with the Adonis function in the R package vegan (Oksanen et al 2019) (999 permutations PerMANOVA) using temperature as an explanatory variable and found no significant difference between temperature treatments (F=1.0562, R_2=0.02344, p=0.32).

Across all samples, a *Fructobacillus* (Leuconostocaceae) ESV was the most abundant bacterium and dominated the communities regardless of temperature treatment (Supplementary Material 1-3). As sequencing data revealed that *Fructobacillus sp.* dominated microbial communities in all samples, we used 16S rRNA gene qPCR to determine absolute abundance to determine if the number of bacteria differed by temperature treatment. We found that there was significantly higher total abundance of bacteria in the

temperature treatment of 32°C compared to the 27 °C treatment (t=-3.804, df=43.97, $p < 0.0001$; Figure 1-5).

DISCUSSION

Temperature treatment affected overall microbial density within nectar which in turn, affected nectar sugar composition and ultimately pollinator preference. Nectar sugars decreased when microbes were present, but overall sugar levels by themselves did not explain bumble bee preference. Fructose levels were lowest with microbes present at the warmest temperature, which agrees with our microbial community data. Our synthetic nectar microbial communities were dominated by *Fructobacillus,* and bacteria were more abundant in the warmer (32 °C) incubation temperature. As its name implies, *Fructobacillus* is a fructophilic lactic acid bacteria that uses fructose as its main carbohydrate source (Endo et al., 2011), indicating that increasing *Fructobacillus* abundance drives the decrease in fructose at the warmest temperature. A caveat to our study is the lack of yeast present in the samples. Yeast is commonly found in floral samples; however, it is not ubiquitous (Jacquemyn et al. 2013). Our lack of yeast detection could be due to inability to culture yeast in the synthetic nectar, or lack of yeast in our field collections.

When given the choice, bumble bees preferred nectar inoculated with a microbial community which was incubated at the lower 27°C temperature over

the higher temperature treatment, suggesting that either the loss of fructose or an overabundance of microbial metabolites influenced bumble bee foraging choices. As *B. impatiens* chose nectar with a microbial community over sterile, more sugar rich, nectar, perhaps microbial metabolites or microbial volatiles are important for bee preference. If this is the case, bumble bees may prefer nectar with microbial metabolites present at lower levels while avoiding nectar with high amounts of microbial metabolites. Whether sugars, microbial metabolites, or interactions between the two drive bumble bee foraging choices needs further study. Overall, our data indicate that the microbial community within nectar is important for pollinator choice and is mediated by abiotic factors such as temperature. As temperatures increases due to climate change, alterations to nectar microbiomes may have adverse effects on pollinator choice.

As pollinators forage for resources they use many mechanisms to choose high quality pollen and nectar. Although high sugar concentrations are important for optimal foraging, the microbial component of nectar is also a significant factor for pollinator choice. For example, honey bees have been known to avoid nectar colonized with the bacteria *Asaia astilbes, Erwinia tasmaniensis,* and *Lactobacillus kunkeei* (Hanshew et al. 2014). However, honey bees are not deterred by nectar colonized with *Metschnikowia reukaufii,* a commonly found nectar-inhabiting yeast (Good et al., 2014). Our study follows a similar pattern of this previous work. Bumble bees did consume more nectar with a microbial community than nectar with no microbes but a higher sugar concentration. This

indicates that these commonly found nectar-inhabiting microbes are potentially advantageous, perhaps giving nutritional benefits to the bee.

We are the first to show that temperature can affect nectar-microbe-pollinator interactions. With an increase in temperature, we saw an increase in the absolute abundance of *Fructobacillus* spp. in the nectar. This greater density of *Fructobacillus* altered nectar chemistry and ultimately pollinator preference, connecting climate change to pollinator behavior as mediated by nectar microbes. Although we only compared two temperatures in this study there is a clear difference in bacterial abundance with temperature, and future studies should look into the effects of a gradient of temperatures on this system. Previous studies have shown that climate change is affecting plant-pollinator mutualisms by causing plant phenological shifts that can disrupt pollinator mutualisms under climate change (Rafferty et al., 2015). As temperatures change and precipitation decreases, not only will floral timing be affected, but also overall landscape composition. Although our lab study does not indicate how pollination success will be affected, we posit that climate change will likely interrupt plant-pollinator mutualisms in ways previously unforeseen by altering nectar-inhabiting microbial communities.

CONCLUSION

Our data show that temperature affects population density of nectar-inhabiting microbes, which in turn alter nectar chemistry and pollinator

preference. We thereby elucidate a connection between climate change, plant- and pollinator-associated microbes, and pollinator behavior. Field studies on these interactions can shed light on whether changes to nectar-inhabiting microbiomes mediated by climate change will influence pollination success and if plants are able to select nectar microbial communities under climate change stress. As our laboratory study may not accurately reflect processes occurring in nature, future studies looking at effects of temperature on nectar-inhabiting microbes and changes in nectar composition *in planta,* in multiple plant species, and in the field, along with studies of foraging behavior of a wide range of pollinators including solitary bees and hummingbirds, and plant fitness will be especially valuable.

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FIGURES

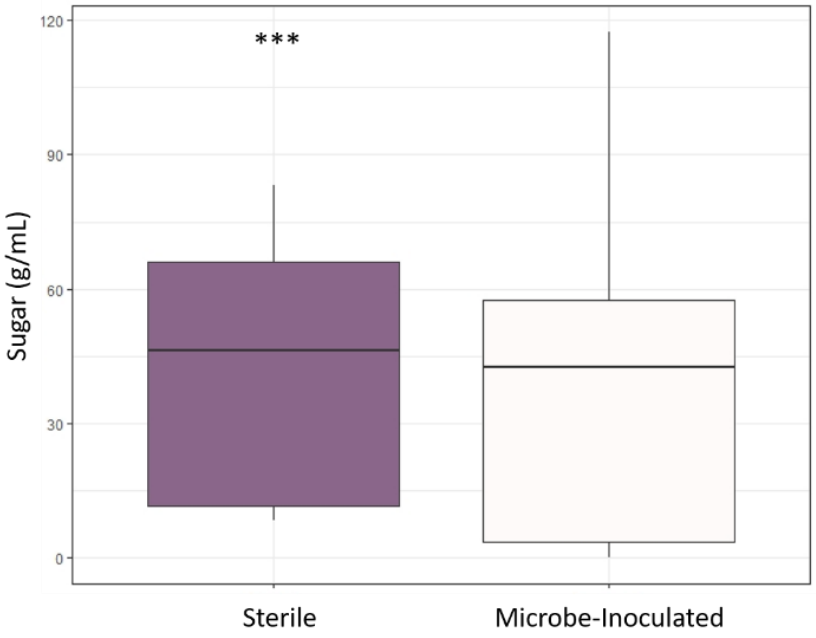


Figure 1-1 Boxplot of Total Sugars
Boxplot showing total sugars (glucose, fructose, and sucrose combined) in synthetic nectar after a three-day incubation period, comparing the treatment (microbe-inoculated nectar) to the sterile controls. There was a decrease in total sugars in the treatments inoculated with microbes compared to the microbe-free nectar (Kruskal-Wallis χ^2= 13.391; p<0.0001. Asterisk (***) indicates statistical significance, purple indicates sterile treatments (controls).

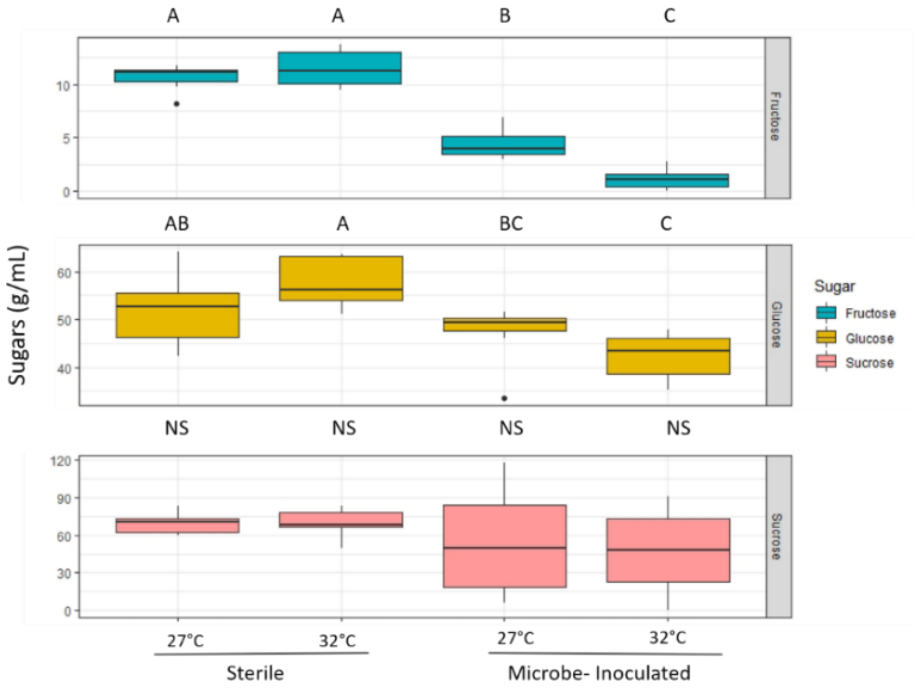


Figure 1-2 Boxplot of Individual Sugars
Boxplot indicating amounts of the three sugars after a three-day incubation. There was an interaction effect of temperature and treatment on fructose levels ($F_{1,36}$=38.8, p<0.0001). More fructose was in the in the 27˚C compared to 32˚C (Tukey HSD, p<0.0001), and significantly more in the sterile nectar compared to nectar with microbes (Tukey HSD, p<0.0001). An interaction effect was seen between temperature and treatment on glucose levels ($F_{1,36}$=10.505, p=0.002) with significant differences in glucose amounts between 32C sterile treatment (Tukey HSD, p=0.003), 27˚C microbe-inoculated treatment (Tukey HSD, p=0.001), and 32˚C microbe-inoculated treatment (Tukey HSD, p<0.0001). There was no significant difference in amount of sucrose between treatments (Kruskal-Wallis χ^2= 2.7792, df = 3, p= 0.4269). Statistical difference is indicated by letters, NS=No Significance.

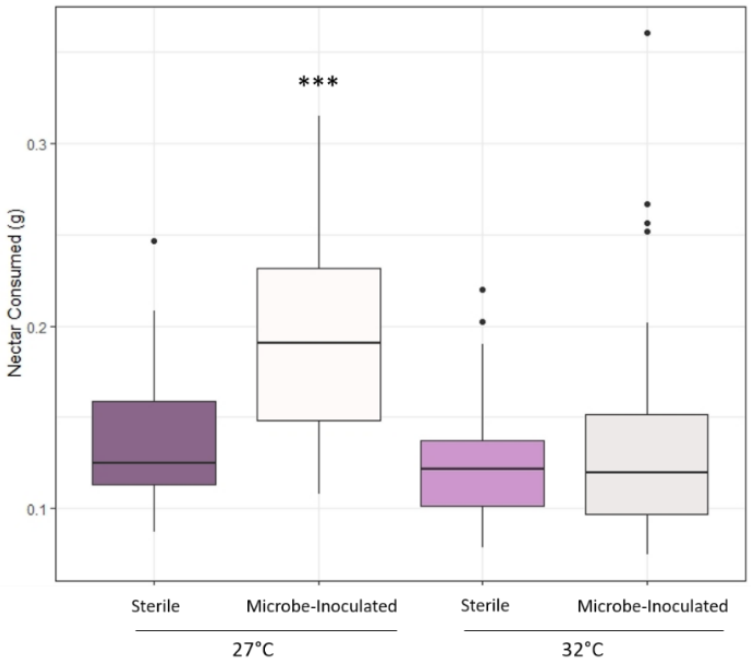


Figure 1-3 Bumble Bee Preference Boxplot
Mass of nectar consumed by bumble bees during the 24-hour choice assay. Bumble bees were giving the choice between four treatments. For the two incubation temperatures, there were a sterile control and a treatment (inoculated with a microbial community). Asterisk (***) indicates statistical significance, purple indicates sterile treatments (controls).

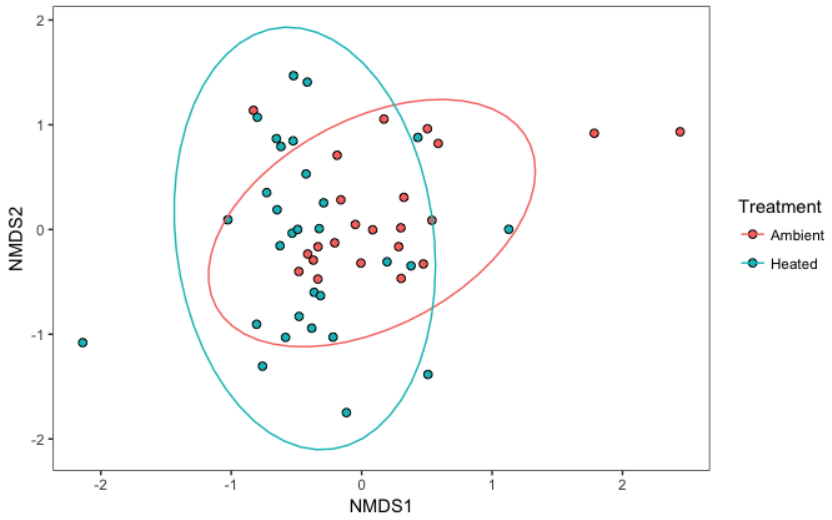


Figure 1-4 Nonmetric Multidimensional Scaling Plot: Ambient v Heated
Nonmetric Multidimensional Scaling plot of the Generalized UniFrac distance matrices of synthetic nectar inoculated with a microbial community and incubated at two temperature treatments. Red points indicate 27°C (Ambient) treatments and blue points denote 32°C (Heated) treatments. Colored ellipses designate 95% confidence intervals around the centroid median of the points. PerMANOVA found no significant difference between the two incubation temperatures (F=1.0562, R2=0.02344, p=0.32).

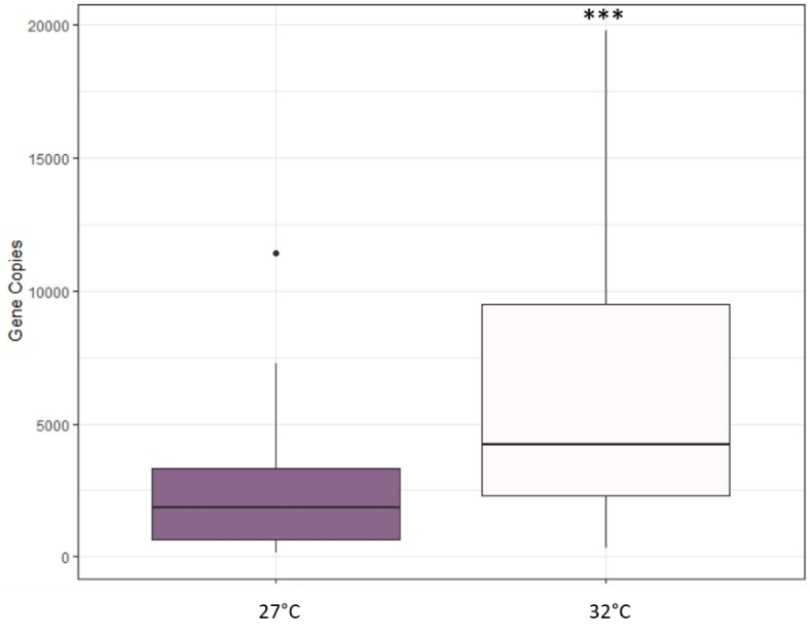
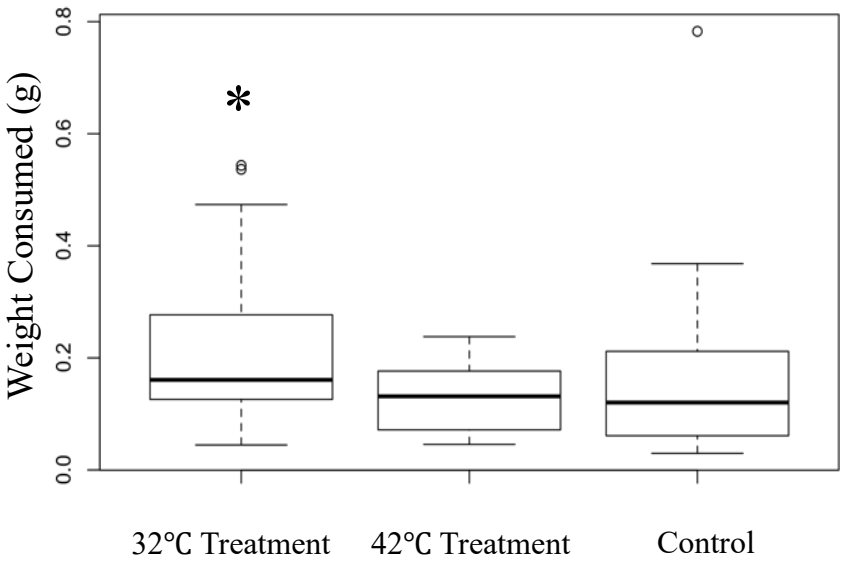


Figure 1-5 *Fructobacillus* qPCR
Quantitative PCR (qPCR) results showing absolute abundance of bacterial cells in each nectar sample of the different temperature treatments; There was an increase in bacterial abundance in the heated treatment ($t=-3.804$, $df=43.97$, $p < 0.0001$). Ambient = 27°C and Heated = 32°C. Asterisk (***) indicates statistical significance.

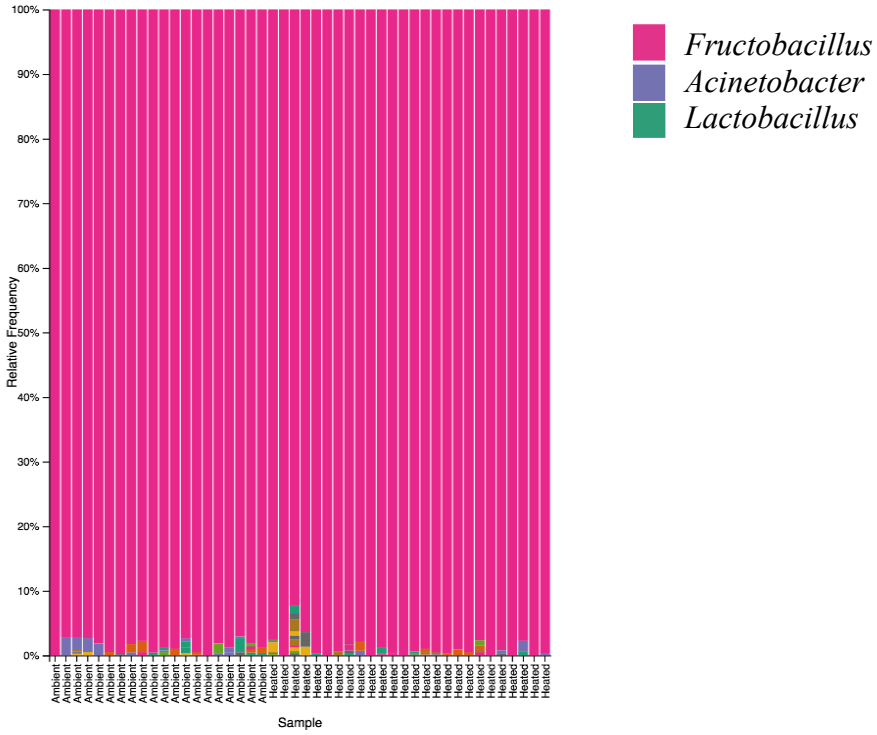
SUPPLEMENTAL MATERIAL

Supplemental Material 1-1. Amino Acid of *Brassica rapa* Table
Results from Texas A&M University Proteomics department's established plant nectar free amino acid HPLC assay.

Amino Acid	mg /ml
ASP	0.271544
GLU	0.313189
ASN	0.811715
SER	0.463781
GLN	1.680674
HIS	0.60187
GLY	0.284765
THR	0.164708
ALA	0.417591
ARG	0.383571
TYR	0.060088
VAL	0.617226
MET	0.05808
TRP	0.331922
PHE	0.093996
ILE	0.169459
LEU	0.20826
LYS	0.208748
PRO	1.266818
Sum:	8.408007



Supplemental Material 1-2 Preliminary Bumble Bee Choice Assay
Bumble bee choice assay from preliminary experiment. Bumble bees were offered a choice between nectar inoculated with microbes an incubated at 32°C, 42°C or sterile synthetic nectar. Bumble bees consumed more nectar with a microbial community and incubated at 32°C ($F = 7.1945$, $p = 0.00118$).



Supplemental Material 1-3 Relative Frequency of Nectar ESVs
Bar graph showing relative frequency of ESVs in synthetic nectar, which were incubated at two temperature treatments: Ambient = 27°C and Heated = 32°C. Legend indicates bacterial genera found in each sample.

CHAPTER 2

Climate change is more likely to affect floral nectar microbial communities via extreme temperature events compared to background warming

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ABSTRACT

Floral nectar contains vital nutrients for pollinators including sugars, amino acids, proteins, and secondary compounds. As pollinators forage, they inoculate nectar with bacteria and fungi. These microbes can colonize nectaries and alter nectar properties including volume and chemistry. Microbial community structure and nectar traits can be influenced by abiotic factors, such as temperatures. In light of current climate change conditions, studying the effects of increased temperature on ecosystem processes like pollination is ever more important. In a manipulative field experiment, we used a passive-heating technique to increase the ambient temperature of a California native plant, *Penstemon heterophyllus*, to test the hypothesis that moderately elevated temperatures will affect nectar traits and nectar-inhabiting microbial communities. We found that passive-heat treatment affected nectar volume but not nectar sugars nor microbial communities. However, a naturally occurring heat wave had large effects on nectar sugars and nectar-inhabiting microbial communities. The initially dominant *Lactobacillus* sp. was replaced by several new colonizers after the heatwave, suggesting that extreme temperatures can reset priority effects. Our study suggests that the quality and attractiveness of nectar under climate change conditions will have implications on pollinator health and pollination success.

INTRODUCTION

Plants entice pollinators with visual displays, floral scents, and food rewards. A main source of pollinator attraction is floral nectar (Heil, 2011). Natural selection for pollinator attraction shapes floral nectar into complex collections of many components. Nectar components vary greatly depending on individual plant and even type of nectary, and these changes can affect pollinator foraging and plant fitness (Cnaani et al., 2006). Nectar secretion may also be mediated by abiotic factors experienced by the plant including water availability, light, temperature, and CO_2 levels (Petanidou & Smets, 1996; Society & Press, 2018; Waser & Price, 2016). The result of these evolutionary and environmental pressures is a dynamic solution that contains not only sugars, but also amino acids, proteins, minerals, secondary compounds, and microbial communities that give nectar scent and color (Adler, 2000; Afik et al., 2014; Hansen et al., 2007; Nepi et al., 2012; Raguso, 2004; Rering et al., 2017).

As pollinators visit flowers to forage for nectar, they insert their microbe-covered mouthparts into the nectaries, effectively inoculating the nectar with bacteria and fungus (Hausmann et al. 2017). Certain microbes are specialized to this ephemeral environment and thrive in the nectary, metabolizing sugars and other resources in the solution (Herrera & Pozo, 2010; Schaeffer et al., 2015; Vannette et al., 2013). During microbial colonization, the composition of nectar properties changes. Microbes alter sugar concentrations, change amino acid and secondary metabolite composition, and release volatile organic compounds

(Rering et al. 2017; Vannette and Fukami 2016; Vannette, Gauthier, and Fukami 2013, Chapter 1). Pollinators consistently choose nectar inhabited by microbes over sterile nectar (Pozo et al., 2012; Vannette et al., 2013), and respond positively to microbial volatiles which can be detected in the floral head space (Rering et al., 2017). Therefore, understanding the interaction between nectar properties and microbial colonization is important for pollinator research. However, nectar production and the microbes associated with the nectaries are not only influenced by plant physiology and pollinator interactions, but also abiotic factors.

Abiotic factors, such as temperature, have become increasingly recognized as important when studying ecosystem processes, especially in light of global climate change. Global surface temperatures have increased 0.85 °C over the past century (Keohane N.O., & Olmstead S.M., 2016). According to high CO_2 emission models, it is predicted that there will be an estimated 5.5 °C rise in the United States by the turn of the century (NRC, 2006). Extreme climatic events, including drought and heat waves, are also predicted to become more common (Diffenbaugh et al. 2017). As the frequency of extreme heat events increase, species interactions and ecosystem functions, such as pollination, may be disrupted (Ockendon et al., 2014). Currently there is a lack of information on how climate change will influence nectar-inhabiting microbial communities and overall nectar attractiveness to pollinators.

In this time of climate crisis and rapid declines in pollinator populations, understanding the effects of warming on nectar-inhabiting microbial communities will give insight into changes in quality of an important food source for insect pollinators – nectar. In a manipulative field experiment, we used passive heating to increase the temperature experienced by California native plant, *Penstemon heterophyllus*. *Penstemon heterophyllus* has long tube-like flowers which are attractive to many pollinators including hummingbirds, Lepidoptera, and many bee species. Because microbes have evolved temperature ranges for optimal growth, we hypothesized that elevated temperature will alter the microbial community structure within nectar. We predicted that as temperatures rise, microbes unable to withstand high temperatures will be outcompeted by more resilient species, changing nectar-inhabiting microbial community structure. We further predicted that as temperatures rise dominant microbial species and plant physiology will alter nectar chemistry, leaving the nectar traits dissimilar from those found under ambient conditions.

MATERIALS and METHODS

Penstemon heterophyllus and study site

Penstemon heterophyllus (Plantaginaceae), the foothill Penstemon, is a drought tolerant, perennial plant that is endemic to the California coastal mountain ranges and Sierra Nevada foothills (Everett, 1950). We used the cultivar “Margarita BOP” which has been developed for Mediterranean-climates

and is drought tolerant. *Penstemon heterophyllus* generally flowers from April to July in Southern California (Everett, 1950). Our experiment took place in the University of California, Riverside's Agricultural Operations (33°57'48.98" N, 117°20'29.30" W).

Experimental design

We set up 25 wooden pallets in "Ortega Park," a shaded section under Jacaranda trees in UCR's Agricultural Operations. Five pallets in a row made up one plot with 5 meters distance between adjacent pallets. We established a total of 5 plots, each 10 meters away from the other. A single pallet consisted of two *P. heterophyllus* plants, one of which was subjected to a passive heating treatment and one was subjected to a non-heated control (Supplementary Material 2-1). All plants were of similar age and had begun flowering when the experiment began. In total, we used 50 plants, 25 in the passive heating treatment and 25 in the control treatment. A passive heating treatment consisted of two 12" x 12" Lexan plexiglass, 2 mm thick sheets attached at the edge to create a 90° angle as described in the International Tundra Experiments (ITEX) (Marion, 1993). We placed 1-gallon potted plants on the pallet and then surrounded the plant with either a heat-treatment or a control. We placed Hobo data loggers (Onset Computer Corporation, Bourne, MA, USA) on the soil of the pots in both passive heating and ambient control treatments to assess difference in temperature based on treatment. To maximize solar heat, we arranged passive heating treatments around the plants in a south facing direction. We

constructed control treatments using Tulle fabric (Joann Item # 15274541) and 18” wooden dowels. These were also constructed to form a 90° angle around the plants to act as a physical block while not altering the floral temperature. We watered each plant daily with 1.5 L of water by hand.

Collection methods

We collected nectar twice a week for six weeks in June-August, 2018, using a 20 µl Biohit® pipette (Swedesboro, NJ) and 20 µl Gilson® pipette tips (Middleton, WI). To quantify nectar production, we recorded volume as we extracted nectar from each flower using volume calibrated pipette tips. We pooled nectar from a single plant into 50 µl of UV sterilized nanopure water in a sterilized and labeled 1.5 ml microcentrifuge tube. We kept pooled nectar on ice in the field and aliquoted each sample upon return to the lab, one portion to analyze nectar sugars and one portion to characterize the microbial communities. To read nectar sugar concentrations we used an Eclipse® hand-held refractometer which reads total percent sugar (Brix%). At the end of the flowering season, we collected capsules from each plant and after a week weighed them in the lab. Once capsule mass was recorded, we dissected the capsules and counted individual seeds.

To extract DNA from the pooled nectar samples, we used the manufacturer protocol for TRIzol® Reagent DNA extractions from Life Technologies (Carlsbad, CA). We included four reagent control ‘blank’ samples that contained no nectar and that we included in all downstream analyses. To

characterize the microbial communities within nectar, we followed the protocols detailed in McFrederick and Rehan (2016), and used a dual-index inline barcoding to prepare samples for sequencing on the MiSeq sequencer (Illumina, San Diego, CA).We used the bacterial 16S rRNA sequence primers 799F-mod3 CMGGATTAGATACCCKGG (Hanshew et al., 2013) and 1115R AGGGTTGCGCTCGTTG (Kembel *et al.* 2014) and the fungal internal transcribed spacer (ITS) primers ITS1F (5'-CTTGGTCATTTAGAGGAAGTAA-3') and ITS4R (5' -TCCTCCGCTTATTGATATGC-3'). Both sets of primers included the Illumina sequencing primers, a unique 8-nt-long barcode, and the forward or reverse genomic oligonucleotide (Kembel *et al. 2014*). We performed PCRs using 10 µL of 2× Pfusion High-Fidelity DNA polymerase (New England Biolabs, Ipswich, MA), 10 µL of ultrapure water, 0.5 µL of each 10 µM primer stock, and 4 µL of DNA, with an annealing temperature of 57°C for 30 cycles. To remove unincorporated primers and dNTPs, we cleaned the PCR products using Ultraclean PCR cleanup kit (MoBio, Carlsbad, CA). To complete the Illumina sequencing construct, we used 1 µL of the clean PCR product as a template for a second PCR, using HPLC-purified primers: CAAGCAGAAGACGGCATAC GAGATCGGTCTCGGCATTCCTGC and AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACG, (Kembel *et al. 2014*). We then normalized 18 µl of PCR product using SequalPrep Normalization plates (Thermo Fisher Scientific, Waltham, MA). We then pooled 5ul of each sample in order to perform another Ultraclean PCR

cleanup on this combined normalized PCR product. We assessed library quality using a 2100 Bioanalyzer (Agilent, Santa Clara, CA). After quality control, we sequenced the libraries using a MiSeq sequencer (Illumina) and MiSeq Reagent kit, version 3 (Illumina), with 2 × 300 cycles, at the IIGB Genomics Core, UC Riverside.

Bioinformatic analysis

To process the 16S rRNA gene sequence libraries and trim low-quality ends off the reads, we used QIME2-2018.6 (Bolyen et al., 2018). Next, we binned our sequences into amplicon sequence variants (ASVs) using DADA2 (Callahan et al., 2016), followed by removing chimeras and reads with more than two expected errors. We used the q2-feature-classifer (Bokulich et al., 2018) trained to the 799-1115 region of the 16S rRNA gene to assign taxonomy to the ESVs and conducted local BLASTn searches against the NCBI 16S microbial database (October 8, 2019). We filtered out ESVs from the resulting feature table that corresponded to contaminants of reagents as identified in our blanks along with chloroplast and mitochondria. We used the MAFFT aligner (Katoh & Standley, 2013) and FastTree v2.1.3 (Price et al., 2010) to generate a phylogenetic tree of our sequences.

Statistical analysis

We used generalized linear mixed models (GLMMs; package lme4) to assess differences in nectar sugar concentrations and seed characteristics by plant through the sampling period in R 3.4.4 (R Core Development Team 2017).

We used nectar sugar concentration or nectar volume as the response variable, temperature treatment as fixed effect, and plant, location in plot nested in plot, and collection date as random effects. To analyze seed characteristics we used LMMS with capsule weight or seed number as the response variable, temperatures treatment as fixed effect and location in plot nested in plot and plant as random effects. We used package lmerTest to compare coefficients of fixed effects. To assess the microbial communities, we used the phylogenic tree developed from our sequences and ESV table for alpha diversity analysis and to calculate UniFrac distance matrices. We used the Shannon Diversity Index and the Kruskal-Wallis test in QIIME2 to analyze alpha diversity. For analyses of beta diversity, we first used betadisper to test for homogeneity of dispersion, then used Adonis (999 permutations PERMANOVA) both in the R-package vegan (Okasanen 2019) with treatment and ambient temperature as independent variables and the Generalized UniFrac matrix as the dependent variable. We also used the Generalized UniFrac distance matrix to perform principal coordinate analysis (PCoA). We performed a non-parametric microbial interdependence test (NMIT) to determine longitudinal sample similarity as a function of temporal microbial composition, in QIIME2 using temperature treatment as the subject. We then performed a feature volatility analysis from q2-longitudinal, to identify indicator species with change in state (ambient temperature).

RESULTS

ITEX passive heating features successfully increased day-time temperatures an average of 1˚C on heat treatment plants. Ambient day-time high temperatures ranged from 28.5-43.5, with a heat wave which began July 7 (collection day 5). Nectar volume was influenced by collection date (GLMM: $F_{1,8}$=5.0581, p<0.001; Figure 2-1) but not temperature treatment (GLMM: $F_{1,44}$=1.3752, p=0.25). Generally, there was more nectar in the ambient treatment later in the season when overall temperatures were higher, but there were some collection days earlier in the season where more nectar was extracted from the heated plants. Nectar sugars were also only influenced by collection date (GLMM: $F_{1,9}$=3.85, p<0.00; Figure 2-1) and not temperature treatment (GLMM: $F_{1,44}$= 0.0972, p=0.7567), with sugar concentrations being higher in the beginning of the season and lowest at the end of the season.

Seed set

Capsule mass was unaffected by temperature treatments ($F_{1,16}$=0.15, p=0.702; Supplementary Material 2-2), as was the number of capsules per plant ($F_{1,20}$=1.1, p=0.304). There were significantly more seeds in the pods of plants that were subjected to the heated treatment ($F_{1,5}$= 4.89, p=0.019; Supplementary Material 2-3), with an average of two more seeds per capsule than the ambient treatment. However, this average is likely due to the handful out outlier capsules that had up to 40 seeds.

Microbial communities

There was a total of 2,157,069 quality-filtered reads with an average of 5,104 reads per sample (N = 284) that clustered into 284 filtered exact sequence variants (ASVs). Through rarefaction analysis, we determined that we had representative coverage of bacterial species diversity at a depth of 2000 reads per sample. We were not able to amplify or sequence fungi from any of our samples. Using the Shannon Diversity Index, we found no significant difference in alpha diversity between temperature treatments (Kruskal-Wallis χ^2= 0.8413, *P* = 0.359). However, there were significant differences between communities in the early season and late season (Kruskal-Wallis χ^2=16.589, *P* < 0.0001), and between communities in the middle of the season and late season (Kruskal-Wallis χ^2 = 20.866, *P* < 0.0001). PCoA analysis on the Generalized UniFrac distance matrix (Figure 2-2) showed clustering by time in both the two-dimensional ordinations. We analyzed the Generalized UniFrac distance matrix of our samples with Adonis and found no significant difference between heating treatments ($F_{1,189}$=0.322, R2=0.00157, p=0.633), however there was a significant effect of ambient temperature ($F_{1,189}$=16.122, R2=0.078, p=0.001; Figure 2-3). For the nectar microbiomes through time, dispersion was significantly heterogenous between early, middle, and late season groups (n=199, p<0.001, $F_{2,196}$= 65.07). The non-parametric microbial interdependence test also found no significant difference by heating treatment (NMIT=1.22, p=0.06).

Although there was no significant difference between microbial communities due to the passive heating treatments, there was significant bacterial turnover throughout the blooming season. Notably, in the beginning of the season the microbial community was dominated by *Lactobacillus, Mesorhizobium,* and *Acinetobacter* (Figure 2-3). After the heat wave there was a loss of *Lactobacillus* and new colonization by *Sediminibacterium*, while *Mesorhizobium* and *Acinetobacter* were still present (Figure 2-3). By the end of the season *Sediminibacterium* dominated the nectar microbial community but with *Mesorhizobium* and *Acinetobacter* still present (Figure 2-3). Feature volatility analysis confirmed these qualitative patterns by revealing that *Lactobacillus, Sediminibacterium, and Acinetobacter* were the indicator species that corresponded with change in temperatures (24%, 6%, and 3% importance respectively).

DISCUSSION

Our data revealed longitudinal shifts in nectar properties and nectar-inhabiting microbial communities across the flowering season. The most drastic shift correlated with an extreme temperature increase, while our passive heating treatment, had no detectable effect on nectar properties or microbial communities. At collection day #5, July 07, 2018, the immediate area experienced a 10 °C temperature spike, with a high of 40˚C recorded on our temperature logger data. Interestingly, it was at this time point that we observed

a shift in microbial communities followed by changes in nectar properties (volume and sugars). Extreme temperature shifts are predicted to become increasingly common under climate change scenarios (Diffenbaugh et al. 2017), and our data suggests that these extreme events may have large effects on nectar microbial communities and nectar chemistry while background increases in daytime temperatures (e.g., 1°C) may have only mild effects.

Nectar properties were not affected directly by temperature treatments but did change across the season. While our data do not allow us to assign causality, shifts in nectar properties coincided with changes in the microbial community. This suggests that changes in nectar properties over time are caused by shifts in the microbial community along with environmental factors, but definitive proof is currently lacking. There is, however, ample documentation that microbial communities can rapidly alter nectar properties (Vannette et al. 2013, Russell and McFrederick, in review), and temperature may therefore influence nectar volume and sugars indirectly through the microbes. As heat waves affect microbial community composition, extreme temperature events could indirectly affect pollination services. Changes in nectar properties influence pollinator preference (Vannette & Fukami, 2016.) and potentially plant fitness (Pozo et al. 2014). Microbial community composition throughout the flowering season is therefore important for pollination services and potentially sensitive to extreme temperature changes.

Nectar-inhabiting microbial communities were significantly affected by environmental temperature. There was a shift in community structure with the loss of the initially abundant *Lactobacillus* ASV immediately following the heat wave. The *Lactobacillus micheneri* clade is commonly associated with plants and pollinators and exhibits optimal growth from 30-35 ˚C and no growth at 40 ˚C and above (McFrederick et al. 2018). The persistence of *Mesorhizobium* and *Acinetobacter* at low levels in the nectar despite the extreme temperatures suggests they are more equipped to handle these temperature spikes. *Acinetobacter* is an environmental bacterium that is commonly found in nectar worldwide and whose abundance is affected by temperature and other environmental factors (Sharaby et al. 2020). Some strains of *Acinetobacter* and *Mesorhizobium* have performed well in incubation temperatures up to 44˚C or when heat shocked at 48˚C in laboratory studies, respectively (Krizova et al. 2015; Laranjo & Oliveira, 2011). Overall, our findings suggest that the extreme heat event led to a loss of *Lactobacillus* from nectaries, which then became open for colonization by *Sediminibacterium* while *Mesorhizobium* and *Acinetobacter* persisted across the entire season, although in low levels.

Priority effects can drive community assembly, especially in nutrient rich and ephemeral nectar-inhabiting microbial communities. In sticky monkey flower (*Mimulus aurantiacus*) nectaries, the microbe that initially established dominance was continually found across multiple floral generations (Toju et al., 2018). Furthermore, temperature fluctuations prevented extinction of late-arriving

species that initially may have been excluded due to priority effects (Tucker & Fukami, 2014). High temperatures have also been shown to have a negative effect on microbial biodiversity. For example, Sharaby et al. (2020) found that slightly elevated temperatures corresponding to elevation significantly reduced bacterial community diversity and evenness.

Our study extends these previous studies by showing that an extreme temperature event can undo priority effects. We initially found communities dominated by *Lactobacillus* which, following normal priority effects, established and persisted for the first couple weeks of sampling until the dramatic heat wave occurred. This heat wave may have facilitated a shift in the microbial communities, allowing for previously unrepresented taxa to colonize the nectar. Once temperature stabilized these new colonists persisted, resulting in dramatically different nectar communities pre- versus post-heat wave. Extreme weather events may therefore disturb and reset priority effects in nectar microbial communities.

Future studies are needed to determine how extreme heat waves affect pollination services. While we were not able to study pollinator visitation pre- and post-heat wave, we did quantify seed set at the end of the experiment as a proxy for pollination success. Capsule counts per plant and capsule mass were the same between treatments, however the passive heat-treated plants had more seeds than the controls. This may be due to a decrease in seed size in the heat-treated plants, as capsule weight did not change. Seed size can affect plant

germination success and potentially plant fitness; it has been well documented that smaller seeds are less competitive than larger seeds (Leishman, 2001). Studies on forbs have observed a positive relationship between seed size and survival from established seedlings to reproduction (Metz et al. 2010). It has also been documented in forbs that there is a negative correlation between seed size and germination success with high temperatures (Yi et al. 2019) along with seed size and drought tolerance (Martínez-López et al 2020). In most cases in forbs, the larger the seeds the more likely the plant is to germinate and survive and the more tolerant the plant is to extreme conditions. If heat treated plants are producing smaller seeds, these seeds could be slower growing and less competitive for resources than larger seeds from the ambient treatment, especially in instances of extreme climatic events. Temperature increase due to climate change may therefore not only affect nectar traits and microbial community but also pollination success and plant fitness.

CONCLUSION

Although our passive-heat treatments had little impact, a heat wave in the middle of our experiment appeared to have large effects on nectar properties and nectar microbial communities. Specifically, a loss in *Lactobacillus* from nectar communities during the heat wave indicated that extreme temperature rise can change microbial community structure, allowing for new community members to colonize. This shift in microbial community may in turn alter nectar traits such as

sugars and volume. Although we were unable to obtain pollinator preference data, this may ultimately affect visitation rates and successful pollination. As extreme weather events including heat waves are predicted to become more commonplace as climate change worsens, our data suggest that climate change could negatively affect plant – pollinator – microbe interactions, and that that extreme shifts in temperature are potentially more important than subtle background changes.

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FIGURES

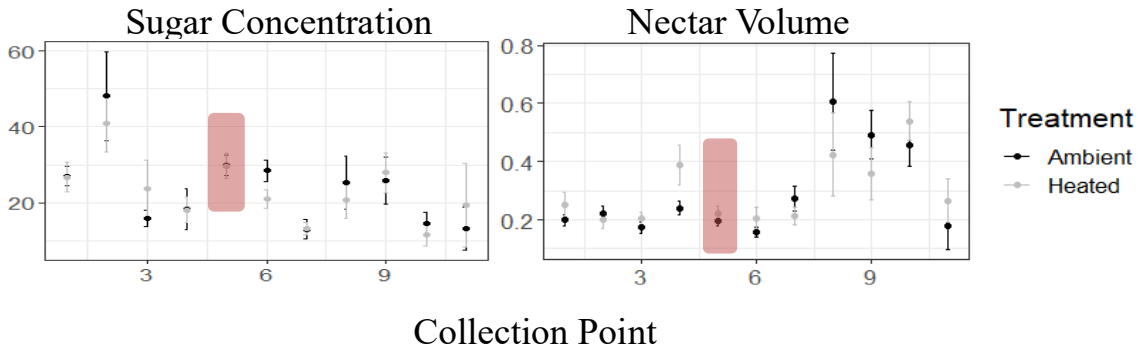


Figure 2-1 Nectar Properties with Passive-Heat Treatment
Nectar sugar concentrations per plant (nectar was pooled for each plant) throughout the sampling period (6 weeks), was significantly influenced by sampling time (GLMM: df= 44, t=-1.718, p<0.000). Nectar volume per flower throughout the sampling period (7 weeks) was significantly affected by sampling time (GLMM: df=44, t=-0.108, p<0.000). Collection point 1-4 were considered "Early" season, as they were pre-heat wave. Collection point 5-7 were considered "Middle" season, which were during the heat wave. Collection point 8-11 were considered "Late" season, as they were post-heat wave. Shaded box indicates beginning of heat wave, which occurred July 7-8 where the maximum temperature was 43.5˚C, a 10˚C increase from the previous day.

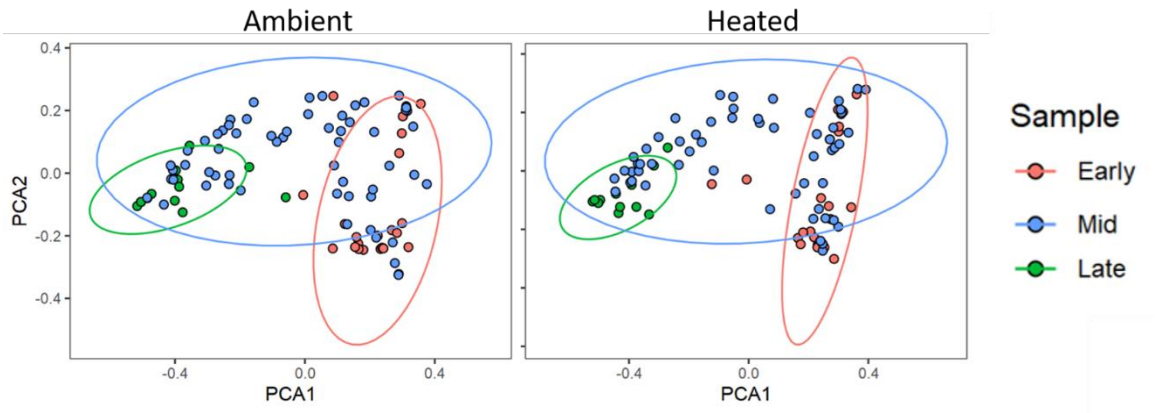


Figure 2-2 Principal Coordinates Analysis Through Time
Principal Coordinates Analysis plot of the Generalized UniFrac distance matrices of microbial communities in *P. heterophyllus* in both treatments through time. Red points indicate the microbial communities at the beginning of collections (June 23, 2018- July 5, 2018), blue points denote the middle collection points (July 7, 2018- July 12, 2018) and green points indicate the late season collections (July 14, 2018- August 1, 2018). Colored ellipses designate 95% confidence intervals around the centroid median of the points. Adonis found significant dissimilarity of microbial communities in *P. heterophyllus* flowers through time ($F_{1,189}$=16.122, R2=0.078, p=0.001).

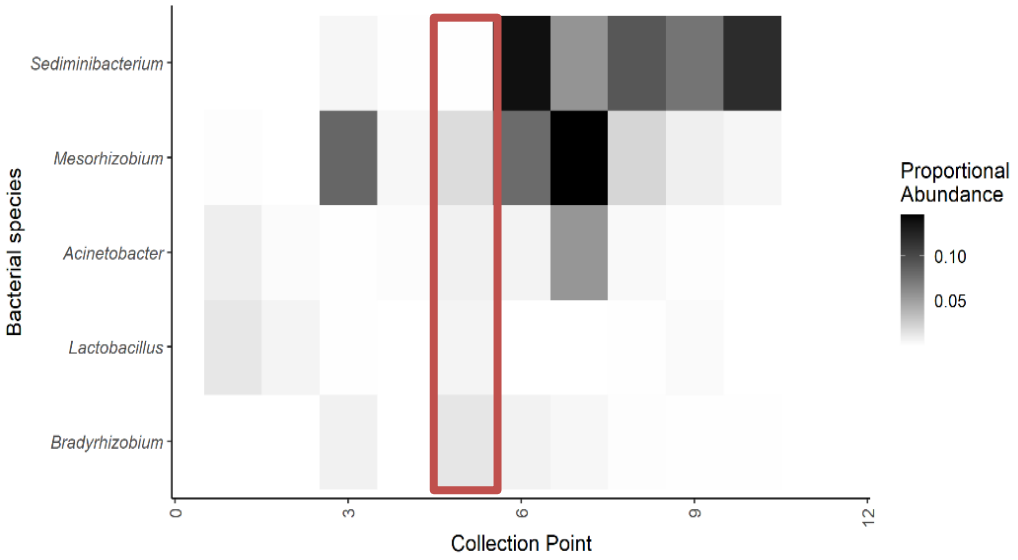
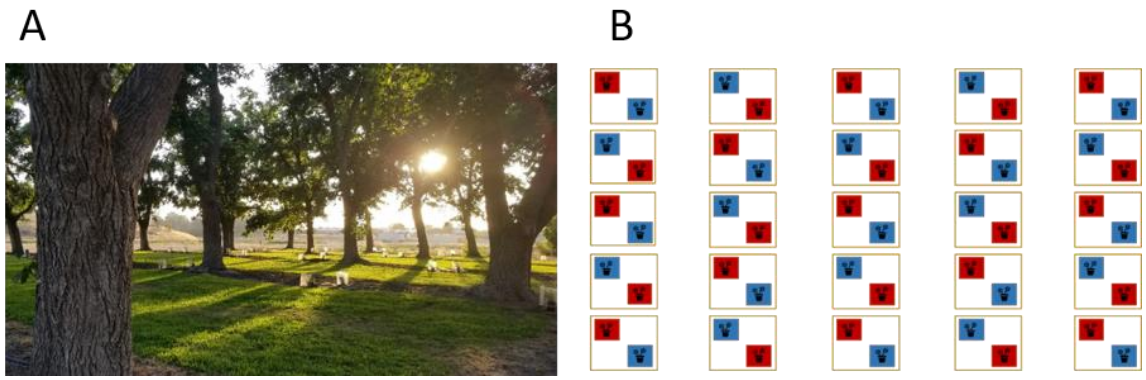
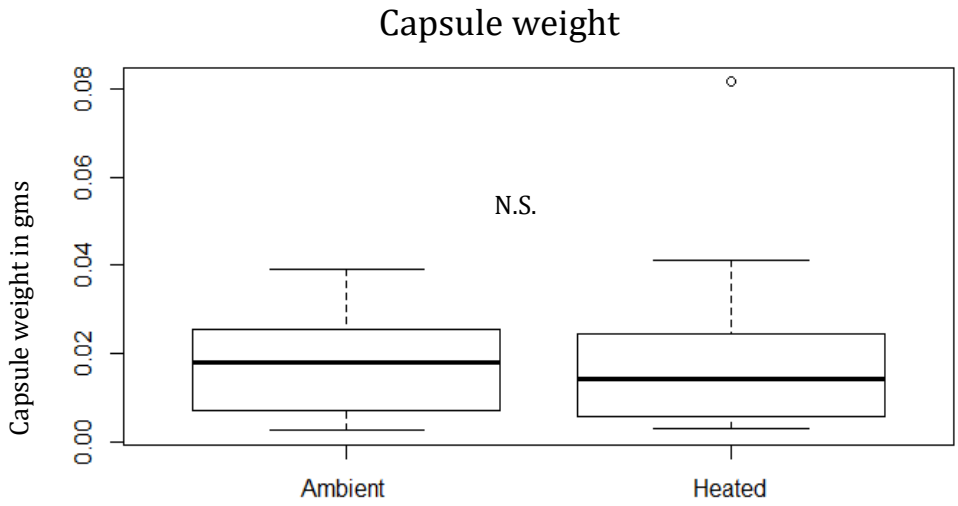


Figure 2-3 Five Most Dominant Microbial Taxa Heat Map
Heat map of five most dominant bacterial taxa early (Collection point 1-4), middle (Collection point 5-7) and late (Collection point 8-11) in the flowering season, a heat wave experience by the region occurred at the first collection day of the "middle" of the season. Adonis found significant dissimilarity of microbial communities in *P. heterophyllus* flowers due to ambient temperature ($F_{1,189}$=16.122, R2=0.078, p=0.001). Orange box indicates beginning of heat wave.

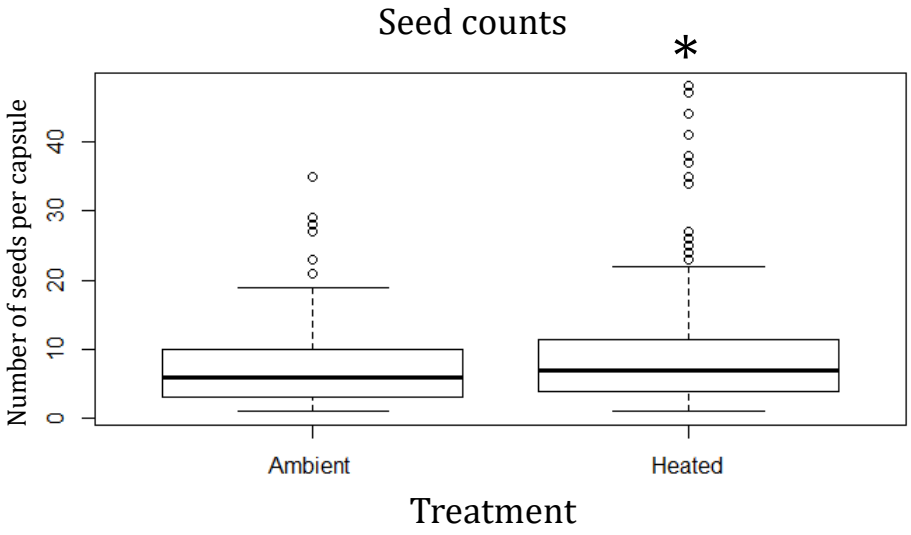
SUPPLEMENTARY MATERIALS



Supplementary Material 2-1 Experimental Design at Ortega Park
A) Image of experimental plot in the Agricultural Operations at the University of California, Riverside. B) Experimental design set up. Brown squares represent a pallet, red boxes represent a heat-treated plant, and blue boxes represent a control plot. In a “column”, pallets were 5m apart, rows were 10m apart.



Supplementary Material 2-2 Capsule Weight
Mass of capsules did not differ between temperature treatments when collected at the end of the flowering period, after the 7-week collection period (t=1.5478, df=558.94, p=0.1222).



Supplementary Material 2-3 Seed Counts per Capsule
Number of seeds within the capsules differed by treatment. There were more seeds in the pods of the heated treatment compared to the ambient treatment ($F_{1,335}$=5.482, p=0.019).

Chapter 3

Elevation affects nectar-inhabiting microbial communities and nectar properties, suggesting that climate change will alter the pollination landscape

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ABSTRACT

Floral nectar harbors a community of microbes may that affect plant and pollinator fitness. These communities are influenced by abiotic factors, such as temperature, suggesting that climate change will alter plant-pollinator interactions. In this study we examine the effects of increased temperature on *Penstemon heterophyllus* nectar using a natural elevation gradient. We collected nectar and observed pollinators along an elevational gradient and characterized nectar-inhabiting microbial communities, nectar sugar concentrations, and pollinator communities at each elevation. Nectar microbes and nectar sugars shifted along this elevational gradient. To our knowledge, we are the first to show that pollinator and nectar-inhabiting microbial communities were correlated along this gradient as well. These results suggest that elevated temperatures caused by climate change may influence the interplay between the nectar microbes, nectar properties, and pollinators, ultimately having negative effects on plant and pollinator communities.

INTRODUCTION

While floral nectar is often considered solely as a reward for pollinators, nectar harbors a community of microorganisms that affects both plant and pollinator fitness. These microbes modify nectar chemistry, nectar volume, and even pollinator feeding behavior (Good et al., 2014; Schaeffer et al., 2016; Vannette et al., 2013, see Chapter 1). Nectar is therefore a miniature ecosystem, yet studies of nectar microbial communities have ignored the abiotic factors that can influence this tripartite interaction. Recent studies suggest that in light of climate change, abiotic factors such as temperature may play a role in how nectar-inhabiting microbial communities assemble, turn-over, and influence plant-pollinator interactions (Tucker & Fukami, 2014). Climate change disrupts plant-pollinator interactions in many ways (Rafferty, 2017), yet how climate change affects nectar microbes mediation of plant-pollinator interactions is an open question.

As nectar-inhabiting microbes utilize nutrients and release secondary metabolites within the nectar there is potential for competition between microorganisms (Álvarez-Pérez et al., 2019; Vannette & Fukami, 2016). For example, the yeast *Metschnikowia* and the bacteria *Acinetobacter* are often found in positive association with each other in nectar. However, in a laboratory experiments, if *Acinetobacter* colonizes nectar first, it will outcompete *Metschnikowia* (Álvarez-Pérez et al., 2019). Such dynamics may be important, as *Metschnikowia* releases volatile organic compounds that are more attractive to

bee pollinators than those emitted by the bacterial genera *Neokomagataea* and *Asaia* (Rering et al., 2017). These alterations to nectar properties may seem slight, but nectar microbes influence pollinator preference, seed set, and plant fitness (Schaeffer et al., 2017; Schaeffer & Irwin, 2014).

With the growing body of evidence that nectar inhabiting microbes are important in understanding pollinator networks (Zemenick et al., 2019) it is crucial to consider the effects of climate change on plant-pollinator interactions. 2020 tied with 2016 as the hottest year on record and predictions suggest that the global temperatures could rise 2˚C by 2065 (Voosen, 2021). Elevational gradients are often used as proxies for climate change in studies of plant community diversity (Bhattarai & Vetaas, 2003; Grytnes & Vetaas, 2002; Vetaas & Grytnes, 2002), plant-pollinator interactions (Hoiss et al., 2015; Ramos-Jiliberto et al., 2010; Richman et al., 2020), and microbial diversity (Hayden & Beman, 2016; Sharaby et al., 2020; Whitaker et al., 2014). Although elevational studies have been used to study nectar microbial communities and pollinator community assembly separately, we are the first to use an elevational gradient to investigate this tripartite interaction.

As pollinator populations decline (Brown & Paxton, 2009) and global temperatures increase (Voosen, 2021), understanding how warming will affect nectar-inhabiting microbial communities will give insight into plant - pollinator conservation. For example, nectar microbes under climate change could become less attractive to floral visitors, which could have consequences to pollination

success and plant biodiversity. Understanding how increased temperatures affect nectar microbial communities and nectar chemistry is therefore a crucial first step in mitigating the effects of climate change on the pollination mutualism. In an elevational gradient experiment, we monitored how temperature affects *Penstemon heterophyllus* nectar (sugar concentrations and total volume), observed pollinator visitation, and characterized nectar-inhabiting microbial communities at four different elevations. Because these microbes have close associations with nectar visitors and have individual optimal growth temperatures, we hypothesized that nectar microbial community structure varies across elevation. We also hypothesized that, because microbes are known to alter nectar properties, a change in microbial communities along an elevational gradient will affect nectar properties and pollinator visitation.

MATERIALS and METHODS

Study site

To characterize effects of elevation and subsequent temperature changes on nectar-inhabiting microbes we used Boyd Deep Canyon Research Station (33˚39’05”N, 116˚22’20”W). This reserve is intentionally designed for climate change research, strategically having reserve land along an elevational gradient up the Santa Rosa mountains. This set-up allows for gradual temperature decreases as elevation increases. Boyd Deep Canyon (BC) is at the base of the mountain range at 180 m above sea level, Agave Hill (AH) is at 760 m, Pinyon

Crest (PC) is 1220 m, and Santa Rosa (SR) is 2286 m above sea level (Table 1). We setup transects of *Penstemon heterophyllus* at each one of these reserve sites. *Penstemon heterophyllus*, the foothill Penstemon, is a drought tolerant plant that is endemic to the California coastal mountain ranges. We used the cultivar “Margarita BOP” which has low-watering needs and long tube-like corolla which are visited by many pollinators ranging from wild bees to hummingbirds (Everett, 1950).

Experimental design

At each of the four elevational sites BC, AH, PC, and SR, we set up two transects of 20 *P. heterophyllus* potted plants for a total of 40 plants at each elevation. We placed each transect at least 500 m apart and each plant within a transect was place 1m apart; we watered each plant daily with 235 mL of water. We surveyed pollinators once a week at each transect for 30 minutes between 10:00am-12:00pm. We collected insect pollinators that landed on *P. heterophyllus* by carefully hand-netting as to not destroy the plant specimens. We put insect pollinators on ice in the field, then froze the specimens at -80 °C prior to identification using *The bee genera of North and Central America* (Michener et al 1994).

We collected nectar from each plant once a week for 6 weeks using a 20 µl Biohit® pipette and 20 µl Gilson® pipette tips. We collected nectar from the first ten flowers that had nectar. As nectar was extracted from each flower, we recorded nectar volume using volume calibrated pipette tips. We pooled nectar

from multiple flowers for each plant into 50 µl of UV sterilized nanopure water in a sterilized and labeled 1.5 ml microcentrifuge tube. We kept nectar on dry ice while in the field and aliquoted samples upon return to the lab, one portion for nectar sugar analysis and one portion to characterize the microbial communities. We read nectar sugar concentrations using an Eclipse® hand-held refractometer which reads total percent sugar (Brix%).

DNA extraction and sequencing

To extract DNA from the pooled nectar samples, we used the manufacturer protocol for TRIzol® Reagent DNA extractions from Life Technologies (Carlsbad, CA). We PCR screened each sample for presence or absence of bacteria and fungi using the following primers respectively: 27F-1492R (Turner et al., 1999, Lane 1991) and ITS1-ITS4 (GARDES & BRUNS, 1993, White et al 1990). We found no presence of fungi, so continued on to follow the protocols detailed in McFrederick and Rehan (Mcfrederick & Rehan, 2016) to characterize the bacterial communities within nectar. We used a dual-index inline barcoding design to prepare samples for sequencing on the MiSeq sequencer (Illumina).We used the bacterial 16S rRNA gene sequence primers 799F-mod3 CMGGATTAGATACCCKGG (Hanshew et al., 2013) and 1115R AGGGTTGCGCTCGTTG (Kembel et al., 2014). The primer set includes partial forward or reverse Illumina sequencing primer, a unique 8-nt-long barcode, and the forward or reverse genomic oligonucleotide (Kembel et al., 2014). We performed PCRs using 10 µL of 2× Pfusion High-Fidelity DNA polymerase (New

England Biolabs, Ipswich, MA), 10 µL of ultrapure water, 0.5 µL of each 10 µM primer stock, and 4 µL of DNA, with an annealing temperature of 57°C for 30 cycles. We cleaned this product using Ultraclean PCR cleanup kit (MoBio, Carlsbad, CA), to remove unincorporated primers and dNTPs. To complete the Illumina sequencing construct, we used 1 µL of the clean PCR product as a template for a second PCR, using HPLC-purified primers: CAAGCAGAAGACGGCATAC GAGATCGGTCTCGGCATTCCTGC and AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACG, (Kembel *et al. 2014*). We normalized up to of 18 µl of PCR product using SequalPrep Normalization plates (Thermo Fisher Scientific, Waltham, MA). In order to perform another Ultraclean PCR cleanup on this combined normalized PCR product, we then pooled 5ul of each sample. We assessed library quality using a 2100 Bioanalyzer (Agilent, Santa Clara, CA). For further size selection, we performed AMPure XP Beads (New England BioLabs Ipswich, MA) purification. We added 60µl AMPure XP Beads (0.3x) to 200µl of pooled Illumina produced and incubated at room temperature for five minutes. We then discarded beads and added 40µl (0.2x original Illumina product), back to the product, for another five-minute incubation on the magnetic stand. After removing supernatant, we washed beads with 200µl of 80% ethanol twice. We then eluted target DNA into 15µl nuclease-free water. After quality control, we sequenced the libraries using a MiSeq sequencer (Illumina) and MiSeq Reagent kit, version 3 (Illumina), with 2 × 300 cycles, at the IIGB Genomics Core, UC Riverside.

Bioinformatic analysis

To process the 16S rRNA gene sequence libraries and trim low-quality ends off the reads, we used QIME2-2018.6 (Bolyen et al., 2018). We were not able to amplify or sequence fungi from any of our samples. We binned our sequences into amplicon sequence variants (ASVs) using DADA2 (Callahan et al., 2016), followed by removing chimeras and reads with more than two expected errors. To assigned taxonomy to the ASVs, we used the silva-138-classifier and conducted local BLASTn searches against the NCBI 16S microbial database (downloaded February 12, 2021). We then filtered out ASVs from the resulting feature table that corresponded to contaminants of reagents as identified in our blanks; we found no chloroplast contamination in our data. To generate a phylogenetic tree of our sequences, we used the MAFFT aligner (Katoh & Standley, 2013) and FastTree v2.1.3 (Price et al., 2010). We used this tree and ASV table to calculate UniFrac distance matrices and for diversity analysis.

Statistical analysis

We analyzed the Generalized UniFrac distance matrix of our microbial samples with Adonis (999 permutations PERMANOVA) and principal coordinate analysis (PCoA), and non-metric multi-dimensional scaling (NMDS). We used the Shannon Diversity Index and the Kruskal-Wallis test in QIIME2 to analyze alpha diversity. For analyses of beta diversity, we first used the “betadisper” function in vegan, (999 permutations) to test for homogeneity of dispersion, then used

Adonis in the R 3.4.4 (R Core Development team 2020) package vegan (Oksanen et al., 2008) with elevation and average temperature as independent variables and the generalized UniFrac matrix as the dependent variable. To test for collinearity between elevation and temperature (Boyd Deep Canyon weather station data) we used a Pearson's correlation test. To analyze pollinator abundance, we used a 2-way ANOVA. For analyses of pollinator diversity, we tested for homogeneity of dispersion, then used Adonis, following the same methods as above, with elevation and temperature as independent variables and the Bray-Curtis distance matrix as the dependent variable. We tested for associations between pollinator abundance and bacterial OTU distance matrices with Mantel tests based on Pearson's product correlation. We used generalized linear mixed models (LMMs; package "lme4Test") with Gaussian error distribution to assess differences in nectar sugar concentrations and nectar volume by elevation. We used Akaike Information Criterion (AIC) values and chi-square likelihood ratio test to conduct model selection, the model with the lowest AIC value for each hypothesis designated the model that we used for analysis. We used nectar sugar concentration as the response variable, elevation as fixed effect, and transect and collection date as random effects. To evaluate differences in nectar volume, we used nectar volume as the response variable, site as fixed effect, and transect and collection date as random effects. To test for the potential of spatial autocorrelation on nectar properties and pollinator communities we used Moran's Index (Moran's I; package "age").

RESULTS

Microbial communities

There was a total of 10,631,877 quality-filtered reads with an average of 23,163 reads per sample (N = 459) that clustered into 2443 filtered amplicon sequence variants (ASVs). Most ASVs belonged to the phylum Gammaproteobacteria (*n* = 2014) and Alphaproteobacteria (*n* = 371) while the remaining 58 ASVs belonged to Proteobacteria. The majority of reads were assigned to Gammaproteobacteria*, Amphiplicatus, Pigmentiphage, Flavobacterium,* and *Zymomonas* with 40%, 26%, 19%, 6%, and 1.5% respectively (Figure 3-1).

Using the Shannon Diversity Index, we found a significant difference in alpha diversity of nectar microbial communities between elevations (Kruskal-Wallis χ^2= 14.81, *P* = 0.005; Figure 3-1). Principal coordinate analysis and NMDS on the Generalized UniFrac distance matrix (Figure 3-2) showed no obvious clustering by elevation. Adonis analysis revealed a significant effect of elevation ($F_{4,287}$=5.56, R2=0.0702, p=0.001), collection date ($F_{1,287}$=6.24, R2=0.01945, p=0.004) and an interaction effect between elevation (Table 3-1) and collection date ($F_{4,287}$=2.87, R^2=0.036, p=0.001, Figure 3-2). Average weekly temperature also affected microbial community beta-diversity ($F_{1,287}$=11.22, R^2=0.037, p=0.002). For the nectar microbiomes for each location, dispersion was significantly heterogenous ($F_{1,40}$=6.2416, p<0.000). There was no correlation

between weekly average temperature and elevation (t = -0.62374, df = 16, p=0.5416).

Pollinator communities

There was a total of 101 pollinators visiting our focal *P. heterophyllus* plants. These pollinators represented 11 taxonomic groups. Although most visitors were bees, we also observed hummingbirds, beetles, flies, and butterflies foraging on focal plants. There were more pollinators at the lower two elevations with 33 at Boyd Deep Canyon and 46 at Agave Hill, while we only observed 17 at Pinion Crest and five at Santa Rosa, the highest elevation sites. There was a significant interaction effect of pollinators by elevation and collection date (ANOVA: $F_{2,30}$=84.88, P=0.002, Figure 3-3). There was no effect of spatial autocorrelation on nectar volume *(Moran's I = −0.355; P= 0.806).* Adonis analysis showed no significant effect of elevation ($F_{3,30}$=0.6, R^2=0.05 P=0.766) or average weekly temperature ($F_{1,33}$=1.62, R^2=0.048, P=0.205) on pollinator community. Mantel tests revealed significant correlations between pollinator and nectar bacterial communities (r=0.06, P=0.004)

Nectar properties

Nectar sugar concentrations (Brix%) varied with elevation (GLMM: $F_{1,151}$= 12.042, p<0.00; Figure 3-4) and temperature (GLMM: $F_{1,151}$= 7.45, p=0.02). A post hoc Tukey test showed that Boyd Deep Canyon (the lowest elevation site) had the lowest amount of nectar sugar ($p < 0.05$). *There was no effect of spatial autocorrelation on nectar sugars (Moran's I = −0.304; p = 0.84).* Nectar volume

was also influenced by elevation (GLMM: $F_{1,139}$=5.29, p=0.002; Figure 3-4) and by date of collection (GLMM: df= 139, $F_{1,139}$=5.29, p<0.000). A post hoc Tukey test again showed that Boyd Deep Canyon differed significantly at $p < 0.05$ from all other elevations. *There was no effect of spatial autocorrelation on nectar volume (Moran's I = −0.168; p = 0.15).*

DISCUSSION

Nectar-inhabiting microbial communities and nectar properties shifted along the elevational gradient, suggesting that elevated temperatures caused by climate change may influence the interplay between nectar sugar, volume, and microorganisms. Both elevation and weekly average temperatures affected nectar microbial communities, suggesting that optimal temperatures may govern microbial assembly within nectar (Sharaby et al., 2020). The highest three sites, which have the most moderate daily high temperatures, consistently had more nectar and higher sugar concentrations compared to the lowest site, suggesting that abiotic pressure on plants may influence nectar production (Parachnowitsch et al., 2019). Differences in nectar properties could be also be influenced by the different microbial communities we reported at each elevation, as individual microbes can alter nectar properties (Vannette & Fukami, 2018). However, pollinator community composition did not significantly differ by elevation, and therefore other drivers such as plant community biodiversity (Bartomeus et al.,

2013) may be more important than temperature for pollinator community assembly.

Pollinator communities in this area are quite diverse (Force, 1990; Jensen et al. 1993). Our results show that there were more pollinators observed in the two lowest sites, with decreasing observations at higher elevations. Although the two middle elevation sites had the most similar temperatures during this sampling period, they also have very dissimilar vegetation community with BC and AH being California desert chaparral, PC transitioning to montane chaparral/woodland, and SR being classified as woodland (Lang 1977). Flower-visitors are more abundant and species-rich in *chaparral* compared to any other type of California vegetation (Force, 1990), which could explain why Agave Hill – a chaparral site with a more moderate temperature, had the highest pollinator abundance. We also noted a correlation between pollinator and microbial communities along the elevational gradient. Pollinator identity and visitation rate may drive assembly of nectar microbial communities (Vannette & Fukami, 2017), and to our knowledge our data are the first to show that pollinator and nectar microbial communities are correlated.

Other researchers have previously investigated pollinator communities along an elevational gradient. Many have found that pollinator species richness and abundance declined with increasing elevation (Hoiss et al., 2015; Marini et al., 2012), along with a deterioration of degree of specialization in plant–pollinator networks (Hoiss et al., 2015). Adedoja et al (2020) also reported differential

species richness with elevation. For example, they found beetles most abundant in their lower sites while bees and flies were most abundant at their higher elevation sites. We also found differential species richness as elevation increased, with beetle and fly pollinators only at Pinion Crest. However, many of these studies are in montane environments, and our study may reveal difference in pollinator assembly across an elevational gradient in the desert.

Microbial community was influenced by elevation and temperature, but also depended on the date nectar was collected. Boyd Deep Canyon, the warmest site, had one of the highest amount of ASV richness, which is contrary to previous studies (Sharaby et al., 2020; Tucker & Fukami, 2014). Boyd Deep Canyon also exhibited large variation in temperature throughout the season and ASV richness could be due to lower early-season temperatures. Agave Hill had the most pollinator visitors but had lower ASV richness, potentially indicating that pollinator identity instead of pollinator species richness influences microbial richness. Although we report that elevation and temperature significantly affect microbial communities, there is still a great deal of unaccounted for variation according to our models, that may be explained by other factors such as vegetation density (Sharaby et al., 2020). As a further caveat, the results from our statistical analyses indicate that differences in microbial community by elevation may be an artifact of heterogenous dispersions between groups. This could reflect a single site, such as Santa Rosa, driving the differences in microbial composition because of greater variation at this site. This variation from

Santa Rosa could explain the statistically significant Adonis results as PERMANOVA is not robust to heterogenous dispersions (Anderson & Walsh, 2013).

We identified six main members of the *P. heterophyllus* nectar-inhabiting microbial community in this area. None of the bacteria identified here have been found in other studies of nectar microbiomes (Álvarez-Pérez et al., 2012), although all are associated with flowering plants, soil, and even heat tolerance (Seo et al., 2005; Wang et al., 2013; Zhen-Li et al., 2014). For example, *Pigmentiphage* is commonly isolated from soil (Wang et al., 2013); *Amphiplicatus,* which was sequenced at all four sites, has strains that have been isolated from hot springs and has been grown in the lab at temperatures between 37–65 °C (Zhen-Li et al., 2014). We also isolated *Zymomonas mobilis*, which is a bacterium that is more efficient at sugar fermentation than many yeasts (Seo et al., 2005). These novel nectar inhabitants suggest that the nectar microbiome may be more variable than expected. We also note that broad surveys of nectar microbial communities are still lacking (Vannette, 2020) and there may be much more diversity that has yet to be discovered.

In a previous study, we characterized the nectar microbial community of the same target plant, *P. heterophyllus*, that we subjected to passive-heat treatments in Riverside, California (Chapter 2, Russell & McFrederick *in review*). We found no effect of these passive-heat treatments on microbial community and posited that such small incremental changes in temperature (~1˚C) were not as

effective at influencing nectar microbes as more extreme temperatures. This hypothesis is supported in our current study as temperature differences between sites ranges from 1˚C to a more extreme 14˚C, and under this more extreme temperature range temperature affected on microbial community structure. Together, our studies (Chapters 2 and 3) therefore suggest that climate change will affect nectar microbial communities via the increased frequency of extreme climate events instead of more subtle background increases in average temperature. In our previous study we found that *P. heterophyllus* nectar was colonized by *Lactobacillus, Mesorhizobium, Acinetobacter,* and *Sediminibacterium*. *Mesorhizobium* and *Sediminibacterium* are not often isolated from nectaries, but are instead associated with plants and soil, indicating likely sources of colonization. However, *Lactobacillus* and *Acinetobacter* are commonly associated with bee pollinators (Vuong & McFrederick, 2019) and nectar microbial communities (Vannette & Fukami, 2018). The difference in these two sets of microbial communities is potentially driven by the environment, as the plants were all the same variant, “Margarita BOP”, and were purchased from the same nursery, although they were purchased one year apart. Microbes are introduced into nectar either by floral visitors or opportunistically moved from the petal or other floral organ (Vannette, 2020), or potentially via air (Lindemann et al., 1982). In either method the microbes must be in the environment already, indicating that the microbial community we found in the present study could be more adapted to the desert environment.

There are several possible explanations for the presence of the same ASVs (albeit at different proportional abundances across elevations) throughout all *P. heterophyllus* flowers in this study. All of these bacteria seem to be heat tolerant. Although average temperature across our study sites and sampling dates ranged from 30- 42˚C all of these temperatures are relatively high. Sharbay et al 2020 found that higher temperatures at lower elevations significantly reduced bacterial community diversity within nectar. They also found that vegetation density was positively correlated with microbial diversity (Sharaby et al., 2020). Nectar microbial communities in our study were collected towards the end of the flowering season in the desert and environmental temperatures were high and vegetation density was low. A combination of low vegetation density and high daily temperatures could have bottlenecked microbial diversity in the area. A caveat to this study is that it was performed late in the flowering season. Blooming in this area starts as early as April, however our sampling started mid-May. Our sampling date was dictated by accessibility to the sites; the highest elevation site has an access road controlled by the county, which was closed due to weather until this time. This late- season sampling could explain the unique pollinator and microbial communities associated with this study.

CONCLUSION

In conclusion, we studied the effects of climate change on plant-pollinator-microbe communities using an elevational gradient. Nectar microbes, sugars, and volume did shift with elevation. Although pollinator communities were not

affected by elevation, they did correlate with nectar microbial communities. This study begins to address yet another way climate change could impact biodiversity. Changes to the nectar-inhabiting microbial community and nectar properties due to temperature increases may have adverse effects on pollinator visitation. As pollinator populations decline and global temperatures increase, further studies on plant-pollinator-microbe interactions under the scope of climate change are especially important.

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TABLE

Table 3-1 Site Description
Description of each site, the sites elevation and average day-time temperature (with standard deviation) during the collection period.

Location of Site	Abbreviation	Elevation (m)	Average day-time temperature
Boyd Deep Canyon	BC	180	38.7˚C ± 4.7
Agave Hill	AH	760	34.2˚C ± 7.8
Pinion Crest	PC	1220	33.1˚C ± 8.8
Santa Rosa	SR	2286	24˚C ± 5.7

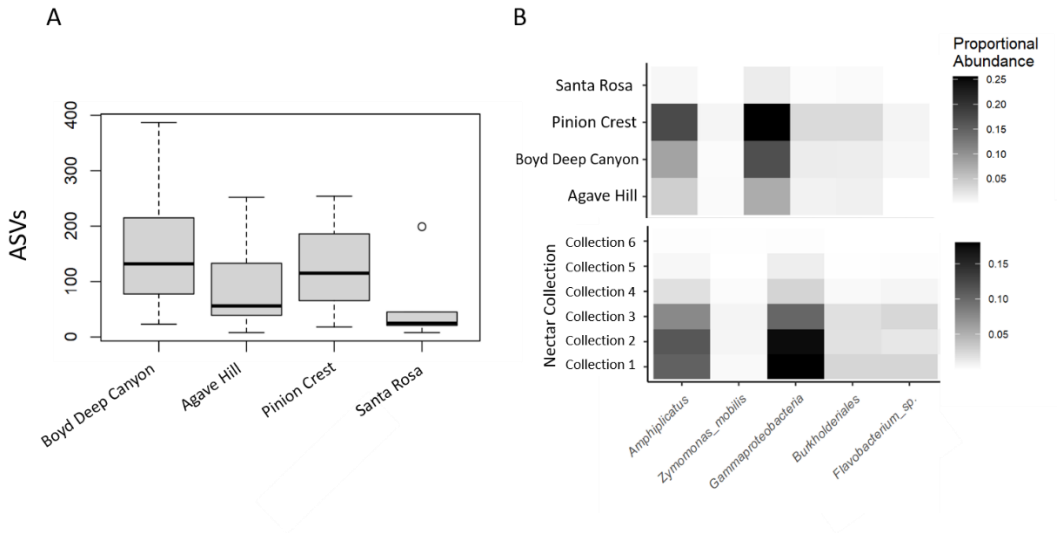

Figure 3-1 Bacterial Species Richness

A) Box plot of species richness of the five most commonly sequenced ASVs at each elevation. Kruskal-Wallis analysis revealed a significant difference in alpha diversity of nectar microbial communities between elevations (Kruskal-Wallis χ^2= 14.81, *P* = 0.005). B) Heat map of five most dominant bacterial taxa (represented by 1.5% of read or more), as seen by location (top) and throughout the season (bottom).

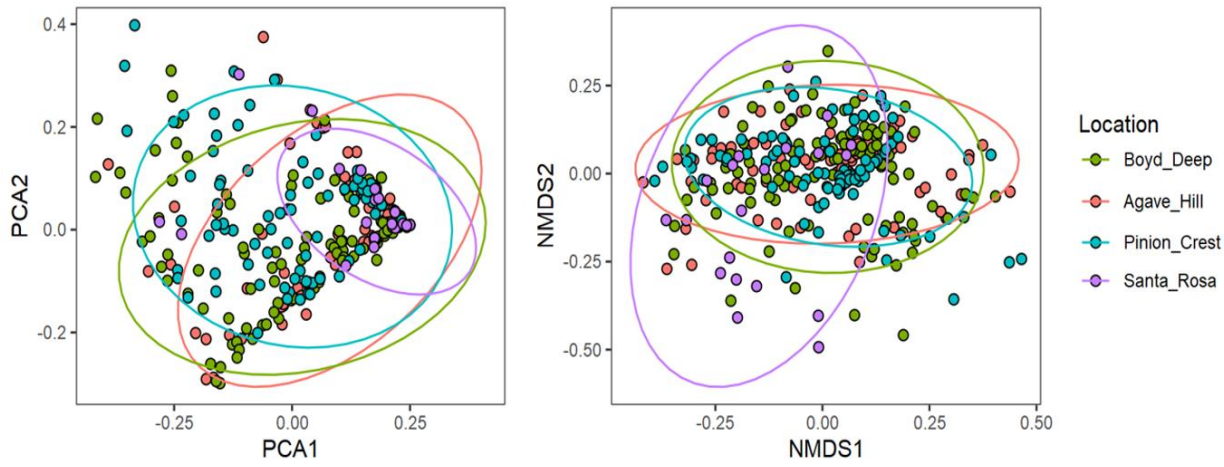

Figure 3-2 Principal Coordinates Analysis and Nonmetric Multidimensional Scaling Plot by Elevation

PCoA and NMDS scaling plots of the Generalized UniFrac distance matrices of microbial communities in *P. heterophyllus* at all four collection sites through time. Colored ellipses designate 95% confidence intervals around the centroid median of the points. Adonis analysis revealed significant dissimilarity of microbial communities in *P. heterophyllus* due to an elevation ($F_{4,287}$=5.56, R2=0.0702, p=0.001) and collection date ($F_{1,287}$=6.24, R2=0.01945, p=0.004) However, dispersion was significantly heterogenous ($F_{1,40}$=6.2416, p<0.000) indicating these results could be driven by clustering of a single site.

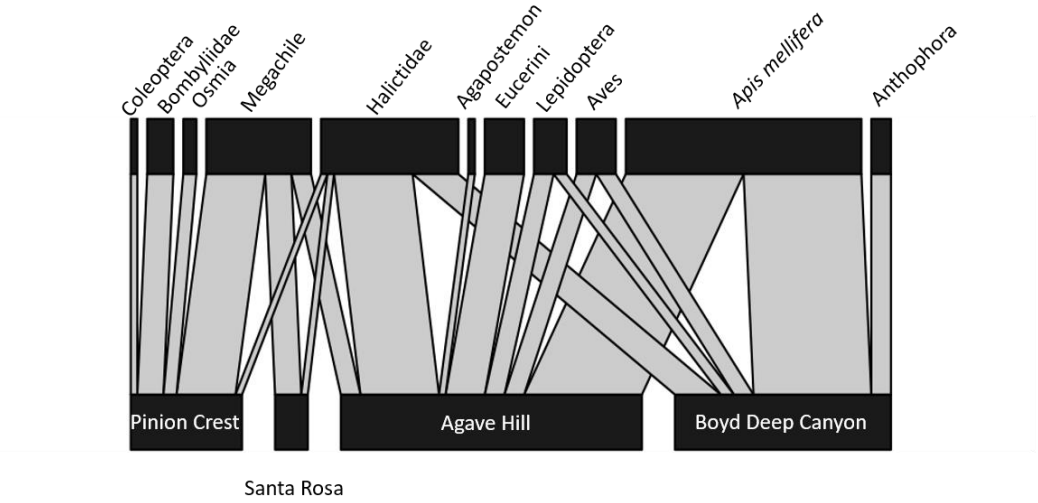

Figure 3-3 Pollinator Interaction Plot with Elevation
Interaction plot depicting the floral visitors (top) at each site (bottom). Floral visitation was influenced by the interaction between location and collection date ($F_{2,30}$=84.88, p=0.002). Pollinator abundance was driven by elevation depending on the collection date, as the latest pollinator observations were mid-summer.

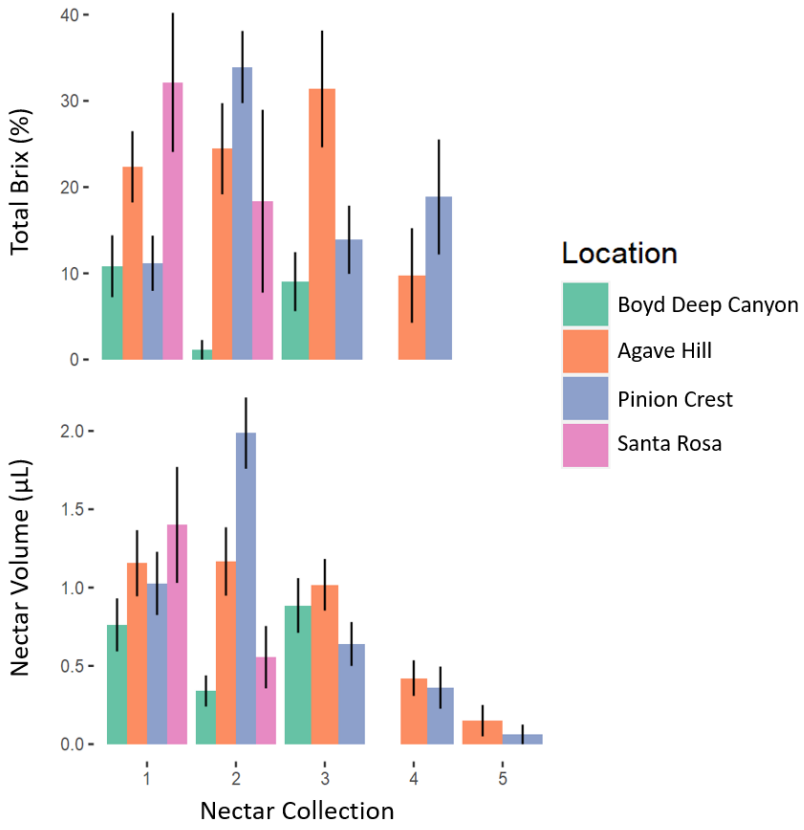

Figure 3-4 Nectar Properties at each Elevation
Nectar sugar concentrations per plant (nectar was pooled for each plant) throughout the sampling period (5 weeks), was significantly influenced by sampling date (GLMM: $F_{1,151}$= 12.042, p<0.001). Nectar volume per flower throughout the sampling period was significantly affected by collection date (GLMM: $F_{1,139}$=5.29, p<0.000) and by elevation (GLMM: $F_{1,139}$=5.29, p=0.002). *Post hoc* analysis indicate that Boyd Deep Canyon, the lowest site, differed from all other sites in both nectar sugar concentration and nectar volume.

CONCLUSION TO DISSERTATION

In a lab experiment, for Chapter 1, I incubated a natural nectar microbial community at different temperatures and assessed the subsequent nectar preference of the common eastern bumble bee, *Bombus impatiens.* The nectar was completely colonized by *Fructobacillus*. Temperature directly influenced the *Fructobacillus* in nectar with an increase of abundance in the warmer treatment. This increase in abundance altered nectar sugars and led to significant differences in pollinator preference. These results indicate that climate change temperatures may make nectar-inhabiting communities less attractive to foraging pollinators.

The passive heating-technique used in Chapter 2 did increase the temperature 1˚C on *Penstemon heterophyllus*. Here I found longitudinal shifts in nectar properties and nectar-inhabiting microbial communities across the flowering season. The most drastic shift correlated with an extreme temperature increase, which altered nectar-inhabiting microbial communities, despite well-known "priority effects." These results suggest that extreme climactic events may be more important than small incremental temperature increases on the nectar-inhabiting microbial community.

I used an elevational gradient in Chapter 3 to represent temperature increase under climate change conditions. Nectar microbes, sugars, and volume did shift

with elevation and temperature. Although pollinator communities were not affected by elevation, they did correlate with nectar microbial communities. Nectar microbes have a dynamic and complex relationship with the plant nectar they colonize and temperature seems to continually interfere with this relationship to a degree, depending on environmental factors.

Overall, nectar-inhabiting microbial communities and nectar properties shifted with temperature in all three studies, suggesting that elevated temperatures caused by climate change may influence the interplay between pollinators and the nectar components: sugars, volume, and associated microorganisms.