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Effects of nutrition on honey bee microbiology, disease occurrence, and hive growth

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EFFECTS OF NUTRITION ON HONEY BEE GUT MICROBIOLOGY, DISEASE OCCURRENCE, AND HIVE GROWTH

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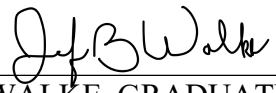

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ABSTRACT

EFFECTS OF NUTRITION ON HONEY BEE GUT MICROBIOLOGY, DISEASE OCCURRENCE, AND HIVE GROWTH

By

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Honey bees (*Apis mellifera*) are major pollinators of many food crops, but unfortunately, population declines are threatening global food security and ecosystem health. Honey bees are under multiple stressors, such as poor nutrition, parasitic mites, and pathogens. Similar to human health, the gut microbiome of the honey bee is hypothesized to affect bee's overall health by supporting host metabolism and immune system. However, it's not clear how stressors impact gut microbiome, and thus health, of bees. Nutritional supplementation could mitigate negative effects of stressors, particularly for bees that don't have access to diverse floral resources. In this study, I conducted a one-year field experiment on 16 honey bee hives at two locations in eastern Washington to evaluate how nutritional supplementation impacts gut bacterial community structure and function, disease occurrence, and overall colony health. The supplementation was mixed in 1:1 sugar-water and mimicked nectar and pollen, consisting of protein, vitamins, and minerals. Control hives were fed 1:1 sugar-water only. To assess gut bacterial community structure before, during, and after feeding treatments, I used 16S rRNA

gene amplicon sequencing on the Illumina MiSeq using primers 515F+barcode and 926R. The bioinformatic programs Quantitative Insights into Microbial Ecology (QIIME) and Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUSt) were used to analyze how nutritional supplementation affected gut microbiome community structure and predicted function, respectively. Additionally, hives were weighed routinely to determine colony growth/productivity. For a subset of timepoints, I screened for *Varroa* mites and microsporidian pathogen *Nosema.*

While supplemental nutrition did not have an overall impact on hive health or gut microbiome, the gut microbiome present at the beginning of experiment correlated with hive survival, suggesting presence/abundance of bacteria present before hives established may have a long-term impact on surviving stressors (i.e. overwintering). Additionally, the gut microbiome was significantly different between hives that survived and those that died at the timepoint before death, further suggesting the microbiome may play a role in hive survival.

With further exploration of bacteria associated with survival (i.e. knockout or inoculation study), a probiotic mixture could be developed and examined for positive influence over hive survival.

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INTRODUCTION

Significance: What Bees Do for Us

The honey bee (*Apis mellifera*) is a major pollinator of important food crops such as almonds, coffee, and many types of beans, herbs, fruits, and vegetables (McGregor 1976, Stein et al. 2017, Garratt et al. 2018, Geslin et al. 2017, Pisanty et al. 2016, Suso et al. 2016, Monasterolo et al. 2015, Benjamin and Winfree 2014, Klatt et al. 2014). These food crops represent a multi-billion-dollar food industry and a major part of global diet; around 75% of agricultural crops rely on pollination by animals such as honey bees (Klein et al. 2007, Gallai et al. 2009). The estimated value for insect pollination in the US is more than $15 billion per year and nearly twelve times that globally (approx. $172 billion) (Gallai et al. 2009). More specifically, almond pollination relies completely on honey bees and almond production is valued around $5.3 billion alone (USDA, National Agricultural Statistics Service 2016).

Without honey bees, many major food crops would be negatively affected. Consequently, the population decline of the honey bee also puts humans in jeopardy in both cultures that rely heavily on small farms kept by families and in industry-owned farms with a prescribed number of managed bee hives per acre.

In addition to our dependence on bees for food crops, bees provide innumerable pollination services to the ecosystem. Honey bees pollinate many types of flowers all over the world; these pollination services are incredibly important for maintaining plant biodiversity (McGregor 1976).

Lastly, honey bees serve as an opportune model organism to study the relationship between the host and its gut microbiome because the honey bee has a relatively simple

community with only eight core bacteria types in the gut (Kwong and Moran 2016, Moran 2015). These eight types of bacteria do not vary with hive location globally and are all culturable, unlike human gut microbiome (Hamdi et al. 2011, Kuwahara et al. 2011, Kwong and Moran 2016) . Additionally, honey bees gain their gut microbiome through social contact, and while humans gain their first microbes during birth, human also gain microbes via social contact; this, in addition to the culturability of their simple gut community, the honey bee is a prime model organism for host, microbiome, and pathogen interaction studies (Kwong and Moran 2016, Moran 2015, Raymann et al. 2017).

Hive Losses Increase Annually

Unfortunately, bee populations are rapidly declining. According to a survey published in 2007, honey bee populations in the United States have decreased by more than half since 1947, from 5.5 million managed colonies to 2.4 million (Mazer 2007). This decline is not due to lack of demand of honey bee pollination services; in fact, pollination needs have risen 300% since 1947 (Potts et al. 2010) . Almond farmers are becoming concerned with honey bee population declines and are quickly trying to find a back-up plan, such as managed native bee hives for pollination (Koh et al. 2018).

This decline in honey bee populations is influenced by a multitude of factors. Honey bees are under several stressors, such as malnutrition, harmful pesticides, side effects of antibiotics, pathogenic bacteria and fungi, and parasitic mites (Raymann et al. 2017, Carina Audisio 2017, Pettis et al. 2013, Erban et al. 2017, Moreira et al. 2012, Le Conte, Yves et al. 2010). These stressors can cause widespread colonial death or colony collapse disorder (CCD) (Dennis and Kemp 2016).

Colony collapse disorder (CCD) is a condition of the colony in which the majority of the worker bees disappear, abandoning their queen behind with the hive (vanEngelsdorp et al. 2009). The actual causation of CCD is unknown, but current research shows its likely a combination of these stressors that weakens honey bee colonies (vanEngelsdorp et al. 2009, Dennis and Kemp 2016).

Since CCD was first described in 2006, populations have drastically declined even further annually (Kulhanek et al. 2017). During 2015-2016, annual hive loss was estimated 40.5% of hives, with winter at 26.9% and summer loss at 23.6%; this is the third year in a row where summer losses are valued close to winter losses (Kulhanek et al. 2017). This year represents one of the lowest hive loss years in the last decade; 2012-2013 estimated 44.8% mortality during winter and 25.4% summer loss with a total annual loss of 45.2%, 2013-2014 estimated 23.7% over winter, 19.8% over summer, and 34.1% annually, and 2014-2015 estimated 43.7% over winter, 43.7% over summer, and 49% annually (Seitz et al. 2016, Lee et al. 2015b, Steinhauer et al. 2014). Short term, hive losses fluctuate; this is thought to be due to fluctuating environmental conditions such as weather between different years (Potts et al. 2010). However, there is a long-term trend of increasing honey bee hive losses (Seitz et al. 2016).

Major Factors of Population Declines

Pathogens

Honey bees are also threatened by many pathogens and parasites; these can be broken into four groups: bacterial diseases such as American foulbrood and European foulbrood (AFB, EFB), fungal diseases such as *Nosema* and chalk brood, parasitic mites (*Varroa*), and viruses like deformed wing virus (DWV). Here I will discuss major diseases of these categories.

Bacteria:

American Foulbrood (AFB) is a highly contagious and widespread disease in honey bees caused by *Paenibacillus larvae*, a spore-forming gram-positive bacterium (Erban et al. 2017). This bacterium infects and kills larvae bees, digests them for nutrients, and releases spores in the process that nurse bees pick up when clearing the brood cell (Pellegrini et al. 2017). Because this can quickly wipe out an entire colony, many beekeepers attempt to use antibiotics such as tetracycline preventatively (Pellegrini et al. 2017).

Fungus:

Nosema is a parasitic microsporidian fungus that infects the gut of honey bees, causing increased mortality, especially in the winter, and is transmitted to other bees in the colony through feces and contaminated food or water (Webster et al. 2004). *Nosema* has shown to suppress honey bee immune system and change foraging behavior; this could implicate that *Nosema* increases susceptibility to other diseases (Moreira et al. 2012, Ferguson et al. 2018).

Mites and Viruses:

Varroa destructor is an invasive species of parasitic mites from Asia (Sammataro et al. 2000). Alone, this mite can cause considerable damage to a honey bee colony (Le Conte et al. 2010, Brettell and Martin 2017). *V. destructor* feeds on the lipids of the bee body and can cause a comprised immune system and, therefore, infections by secondary pathogens (Hamiduzzaman et al. 2017, Ramsey et al. 2019). Additionally, *V. destructor* acts as a vector for a wide range of devastating of viruses such as Deformed Wing Virus (DWV) (Le Conte et al. 2010). Combined, *V. destructor* and associated viruses are grievously destructive and cause the death of millions of honey bee colonies worldwide (Brettell and Martin 2017).

Environmental changes

Climate change:

As climate changes globally to become warmer and drier, honey bees face several challenges (Le Conte and Navajas 2008). Late spring and early summertime are peak foraging seasons (Núñez 1976). However, with this time of year getting hotter, honey bees have to dedicate more time to collecting water to bring into the hive to cool by evaporation (Le Conte and Navajas 2008). This is problematic because spring and summer are also becoming drier. If there is not a good water source near the hives, bees may need to fly for miles to collect enough water to effectively cool the hive. This leaves less time and bees to forage for nectar and pollen. As a result, the hive ends up with less food hoarded for wintertime and can lead to overwintering starvation.

A hot and dry climate can also halt nectar flow (Le Conte and Navajas 2008). Not only is this an issue for food supply, but this can also cause an increase in yellow jacket pressure because they, too, are desperate for a new food source; yellow jackets are aggressive honey robbers and can predate on honey bees (Le Conte and Navajas 2008).

Poor nutrition:

Bees require a complex combination of nutrients and forage for pollen, nectar, and resin to fulfill this nutrient need (Wright et al. 2018). Adult bees need carbohydrates and sugars found in nectar for foraging and thermoregulation. Nectar is stored in the hive long-term as honey. Larvae and developing bees need protein, fats, vitamins, and minerals, mainly found in pollen; however, both nectar and pollen contain a mixture of micronutrients (e.g. vitamins, minerals) and antimicrobial compounds (Wright et al. 2018). Pollen is stored in the hive as bee

bread. Plant resin is collected to make propolis, a material used for increased structural support and its antimicrobial properties within the hive (Wright et al. 2018).

Poor nutrition is mainly the result of a decrease in biodiversity of plant life (Brown and Paxton 2009). This decrease in biodiversity is caused by a combination of environmental factors (e.g. climate change, urbanization, deforestation, farming monocultures, etc) (Foley et al. 2005). In addition to decrease in biodiversity, dry weather due to climate change can halt nectar flow and cause a decrease in nutrient availability (Le Conte and Navajas 2008).

Besides the obvious concern of starvation, poor nutritional availability can alter other factors of bee health. For example, honey bees that received pollen from a single source (mono-floral) were shown to have a significant decrease in immunocompetency in comparison to bees fed poly-floral pollen (Alaux et al. 2010). This is a significant problem when considering the magnitude of hives that are leased to pollinate monocultures of almonds and other crop foods. Furthermore, bees prefer fresh pollen sources over artificially preserved pollen sources (Anderson et al. 2014). A separate study showed a potential mechanism for this preference: pollen allowed to age can cause dysbiosis, decreased development, and increased mortality (Maes et al. 2016). Nutrition, commensal gut microbes, and general health of honey bees seem to be highly connected in relation to survival and susceptibility to disease.

Gut Microbiome in Relation to Health and Nutrition

We know microbes are essential to nearly all animal life (Zilber-Rosenberg and Rosenberg 2008). Commensal microbes play many different roles in health overall such as protection against pathogens (directly by taking up space and producing antimicrobial compounds and indirectly by stimulating host immune system), protection from allergies and

auto immune diseases by training the immune system, metabolizing food products into usable nutrients, producing chemicals to stimulate metabolic rates, detoxifying metabolic biproducts, and synthesizing vitamins and other molecules such as neurotransmitters (Dorrestein et al. 2014, Flint et al. 2012).

Just as we have found it so in animal health, the gut microbiome of honey bees affects the honey bee's overall health, immune system, and efficiency as a hive (Kwong and Moran 2016, Engel and Moran 2013). Worker honey bees have eight core bacterial groups living in their gut that do not change with hive location globally (Kwong and Moran 2016, Schwarz et al. 2015, Hamdi et al. 2011). Honey bee gut microbes are transmitted through socialization with other bees in the colony or picked up from outside environments (i.e. flowers) (Kwong and Moran 2016). Because of these similarities between human and honey bee gut microbiome and due to the simple community that colonizes honey bees, honey bees can be used as a model organism for better understanding host-microbiome interactions (Zheng et al. 2018).

The eight core bacterial groups consist of *Bartonella apis*, *Parasaccharibacter apium*, *Frischella perrara*, *Snodgrella alvi*, *Gilliamella apicola*, *Bifidobacterium spp.*, and two groups of *Lactobacillus* (Firm-4 and Firm-5). *G. apicola* is the most abundant bacterium in honey bee guts and is important for metabolism of important nutrients; this bacterium is able to degrade and process carbohydrates, including pectin found in pollen cell walls and complex carbohydrates that would otherwise become toxic for bees (Kwong et al. 2014, Zheng et al. 2016). *S. alvi* also aids in metabolism of nutrients; it processes the bi-products from *G. apicola's* metabolism of complex carbohydrates. Additionally, *S. alvi* secretes a biofilm that prevents occupation of the gut by potential pathogens (Kwong and Moran 2016). *B. apis*, first described in 2016, has an

unknown function to honey bee health (Kekerova et al. 2016). *P. apium* and *F. perrara* are both bacteria that can be found within the hive environment; function of *P. apium* is not well understood but *F. perrara* is believed to be an opportunistic pathogen correlated with a decrease in *S. alvi*, increased *Nosema* infection, and immune-linked scabbing of the midgut (Maes et al. 2016, Engel et al. 2015, Engel and Moran 2013). *Bifidobacterium* and *Lactobacillus* are also found in human gut microbiome and are commonly found in probiotic mixtures; in honey bees, *Bifidobacterium* is associated with an increase in host-derived signaling molecules such as prostaglandins, potentially stimulating inflammation or regulating growth hormones (Kesnerova et al. 2017, Zheng et al. 2017). Lastly, *Lactobacillus* is a lactic acid fermenter that is helpful for metabolism of nutrients and secretion of enzymes and vitamins (Kesnerova et al. 2017).

Because so many bacterial groups of the honey bee core gut microbiome assist with metabolism of nutrients, disturbance to this core (i.e. dysbiosis) can potentially facilitate poor nutrition conditions by lack of metabolic support and nutrient synthesis. Furthermore, dysbiosis has been shown to occur with each stressor (described in above section) that honey bees face (Hamdi et al. 2011, Raymann et al. 2017, Erban et al. 2017, Moreira et al. 2012, Kakumanu et al. 2016, Maes et al. 2016).

Gut Microbiome supports metabolism and synthesizes nutrients

Microbes have been shown to digest materials that bees are not able to, such as the cell wall of pollen spores and sugars that can be toxic to bees when built up in the body (Kwong and Moran 2016, Zheng et al. 2016, Engel et al. 2012). Honey bee gut microbiome also assists with metabolism of saccharides, a major part of honey bee diet found in nectar and honey (Lee et al.

2015a). Furthermore, the gut microbiome has been found to promote host weight gain through metabolism of nutrients and hormone-mediated changes in the host's metabolism (Zheng et al. 2017). Specific bacterial groups such as *Bifidobacterium asteroides* have been identified to promote these hormone-mediated changes while groups such as *Lactobacilli* are associated with high metabolic output; both are associated with host weight gain (Kesnerova et al. 2017).

Honey bee gut microbes secrete important micronutrients such B vitamins that support metabolism (Wright et al. 2018). Additionally, gut microbes help with processing foraged materials such as nectar and pollen into more nutritious materials for long-term storage. Specifically, *Lactobacillus* regurgitated with pollen and nectar into storage cells enrich the food with vitamin K (Arathi et al. 2018).

Gut Microbiome supports immune system

Gut microbiome helps educate immune system to better identify future pathogens and amount a good immune response (Kwong et al. 2017). This is thought to be similar to the education of mammal's immune system by pathogen-associated molecular patterns (PAMPs), suggesting convergent evolution of the immune system between insects and mammals, although the mechanism in insects is still poorly understood (Kwong et al. 2017, Schmid-Hempel 2005). Presence of commensal microbes also helps stimulate the production of antimicrobial peptides (AMPs) and other major host immune responses (e.g. lysozyme, proteolytic, hydrolytic enzymes, etc) (Kwong et al. 2017, Schmid-Hempel 2005). Interestingly, the majority of these immune response molecules are produced in the fat bodies of the bees, which has remarkable implications regarding immunocompetence during *Varroa* mite

infestation now that we know the *Varroa* mite feeds mainly on fat bodies (Schmid-Hempel 2005, Ramsey et al. 2019).

Several studies have shown a correlation between higher infection rates of various pathogens and an alteration gut microbiome, either in number or species. For example, both diet-related and pesticide-related dysbiosis have been shown to correlate with increased *Nosema* susceptibility and mortality rates (Pettis et al. 2013, Maes et al. 2016f). The mechanism of protection that the gut microbiome confers to honey bees against *Nosema* is not yet understood, but since *Nosema* induces immunosuppression in honey bees, it is likely that the gut microbiomes help stimulate the host's innate immune system to recognize *Nosema* (Glavinic et al. 2017). Another explanation could be provided by two core bacterial groups, *Snodgrassella* and *Gilliamella*. These two bacterial groups are both sugar fermenters, facilitating in host's metabolism of nutrients, but they may also confer pathogen resistance by secreting a biofilm within the honey bee midgut, likely preventing gut pathogens from growing and causing disease (Kwong and Moran 2016, Engel et al. 2012).

Combined Effects of Poor Nutrition and Dysbiosis

Poor nutrient availability or quality can lead to a decrease in available energy, time, and resources for honey bees. This creates an intricate feedback loop with many factors. Malnourished bees would not have the energy required for sufficient immune response to pathogens (Alaux et al. 2010). Bees would not have the nutrients needed to develop to a proper body size, which creates weak bees that often are cannibalized as larvae or die under frequent stressors (Brodschneider and Crailsheim 2010). Productivity would potentially be decreased among all social castes without energy (queen does not lay enough brood, nurse bees do not

feed enough bees, cell cleaners fail at keeping up basic hygiene standards, foragers do not collect enough food, guards cannot keep out robbers, etc), although productivity and nutritional stress has not been explored exclusively (Wright et al. 2018, Brodschneider and Crailsheim 2010). The hive would not be able to properly regulate temperature in the hive; in the summer, bees would not have the time or energy to collect water to cool the hive without proper nutrition and in the winter, bees would not have the energy to "shiver" wings to warm the winter cluster (Wright et al. 2018). Poor nutrition can also lead to dysbiosis; gut microbes therefore will not be able to supplement metabolism, stimulate the host immune system, or inhibit pathogens. Therefore, dysbiosis feeds back into this cycle of malnutrition and susceptibility to disease (Maes et al. 2016). All of these factors work together and cause enough stress for a colony to collapse.

Nutritional Supplementation

Nutrition supplementation could be the solution to breaking this cycle of malnutrition and disease. Beekeepers commonly feed their hives a sugar-water mixture during times of nutrient stress, but a simple sugar mixture doesn't include all the complex micronutrients that nectar contains (Wright et al. 2018). Similarly, a common pollen substitute that contains only protein cannot provide for all nutritional needs (DeGrandi-Hoffman et al. 2016). For a nutrient supplement to protect and fortify bees against the variety of stressors they encounter, the supplement needs to contain all the micronutrients they would gather from nectar and pollen in the wild (Dolezal and Toth 2018). There has been some research done regarding health benefits of nutritional supplements, but no one nutritional supplementation is the same between these projects (Dolezal and Toth 2018). With this in mind, as a broad statement,

nutritional supplements have shown to mitigate effects of some of the many of the stressors bees face.

Protein supplementation in general seems to increase immunocompetency in insects (Lee et al. 2008). For example, one study showed that an amino acid and vitamin supplementation stimulated the immune system against *Nosema*, quantified by measuring immune-related peptides and number of *Nosema* spores (Glavinic et al. 2017). Another study showed that a nutritional supplement with pollen applied over winter increased queen productivity and the amount of brood laid in early spring in comparison to a nutritional supplement without pollen (Ricigliano et al. 2018).

How does nutrient supplementation affect the gut microbiome?

In human systems, we know that healthy, diverse diets correlate with a functionally diverse microbiome (Flint et al. 2012). A functionally diverse microbiome would be expected to be beneficial in regard to disease susceptibility either (1) indirectly by increasing nutrient uptake to support a more robust immune system or (2) directly by increasing bacterial groups that inhibit pathogens and parasites and/or educates the immune system (Dorrestein et al. 2014).

There are only a few studies that examine at how various nutritional supplementation changes the structure of the gut microbiome in honey bees. For example, one study tested different syrup mixtures against honey and found an increase in *Rhizobiales* (the order that contains *B. apium*) and *Bifidobacteria* in honey and wheat starch syrup treatments in comparison to standard sugar water (D'Alvise et al. 2018). Another study compared foraging draught and supplemental flower forage during winter months and found a slight difference in

several bacterial taxa at one timepoint (Rothman et al. 2018). Outside of honey bees, a recent study using frogs showed that dietary stress early on in life only temporarily affected host microbiome structure but permanently affected susceptibility to parasites (Knutie et al. 2017). No honey bee microbiome-nutrition studies that I am aware of also include whole bacterial community functional diversity.

OBJECTIVES

This study aimed to understand the effects of nutrient supplementation on several factors of honey bee health. The first chapter will focus on factors of overall hive health, such as honey production and hive growth, disease susceptibility, and hive survival. The second chapter will focus on how nutrient supplementation affects the structure and function of the honey bee gut microbiome.

The first objective of chapter one is to test the effects of nutrient supplementation on hive efficiency and growth. My hypothesis was that supplementing hive colonies with synthetic nectar and pollen would increase honey production and population growth because bees would have direct access to nutrients that help build up honey stores, support larvae growth, and potentially minimize time and energy foraging for external food resources.

My second objective of chapter one was to test the effects of nutrient supplementation on disease occurrence and survival rates. In fall and spring, I measured *Varroa* mite counts and *Nosema* levels in the hives to determine correlation of disease occurrence with treatment. Based on the BeeInformed US survey mentioned in the introduction, I expected hive losses over summer and winter, so I compared nutrient supplementation to survival rate to determine if supplemental nutrients would help boost overall hive strength and survival (Lee et al. 2015a). I hypothesized that hives fed with synthetic nectar would have a decreased susceptibility to disease (i.e. *Varroa* mites, *Nosema*) in hives fed synthetic nectar because additional amino acids and vitamins have been shown to stimulate immune function and decrease *Nosema* spore numbers (Glavinic et al. 2017). I also expected that hives fed synthetic nectar would have higher

survival rates during times of environmental stress (i.e. overwintering) because these hives would have more energy stores and a strong population in comparison to sugar-water control.

In chapter 2, the goal was to understand the effects of nutrient supplementation on the structure and function of honey bee gut microbes. To do so, I examined changes in overall gut microbiome structure within and between samples as well as changes in the presence and abundance of key microbes that differ in previous nutrition and disease studies and are theorized to provide beneficial functions, such as decreased disease susceptibility either (1) indirectly by increasing nutrient uptake to support a more robust immune system or (2) directly by increasing bacterial groups that inhibit pathogens and parasites (i.e. *Lactobacillus*, *Snodgrasella*, *Gilliamella*, *Frischella*) (Kwong and Moran 2016, Lee et al. 2018, Lee et al. 2015a, Zheng et al. 2016, Kwong et al. 2017). I also compared changes in these key microbes against survival rates and hive productivity (see chapter 1 objectives). Lastly, I used predictive metagenomics to evaluate how the theoretical function of the microbiome changes across the experiment (Langille et al. 2013). I hypothesized that supplementing hive colonies with a mixture of sugar, water, and a select group of amino acids and vitamins that mimic naturally occurring pollen and nectar would have a beneficial effect on the function of the gut microbiome of honey bees as opposed to hives supplemented with solely sugar and water, specifically increasing microbial gene functions related to increased metabolism of nutrients and competition with pathogenic microbes.

CHAPTER ONE: EFFECTS OF NUTRITION ON HONEY BEE HIVE HEALTH

Methods

For chapter one, I hypothesized that supplementing hive colonies with a mixture of sugar, water, and a select group of amino acids and vitamins that mimic naturally occurring pollen and nectar would one, increase honey productivity and population growth and two, decrease susceptibility to diseases such as *Nosema* and *Varroa destructor* mites.

Hive set up & Treatments

To test these hypotheses, I established apiaries at two local study sites (one site on the Eastern Washington University campus in Cheney, WA, and one site on private property in Four Lakes, WA) in April 2018. The honey bees were Carniolan bees from Olivarez Honey Bees, Inc. in northern California and stored in 10 frame Langstroth box hives. Honey bee hives at both locations were fed a supplemental mixture of either 1:1 sugar-water ('sugar' treatment) or 1:1 sugar-water plus a select group of protein, vitamins, and minerals (synthetic nectar and pollen treatment, referred to as 'nectar' treatment) by adding the liquid mixture to a 1-gallon feeder frame located inside each hive (Figure 1.1). Each site had 8 hives, with 4 fed sugar and 4 fed nectar (n=16 hives total) (Table 1.1). The synthetic nectar contained nutrients commonly found in nectar and pollen (Table 1.2) (Brodschneider and Crailsheim 2010). Because of the diverse mixture of nutrients in the treatment, this study did not strive to determine which particular nutrient affects bee health, but rather how the combination of nutrients that mimic natural pollen and nectar affect bee health.

Weekly feedings of hives occurred throughout the spring and fall seasons, from initial set up in April 2018 to October 2018 (Table 1.3). The duration of feeding depended on nectar flow and

temperature. Specifically, spring feeding began when temperatures were steadily above 45^{o}F and end when flowers began to bloom (approx. April through June). Fall feeding began when there was a decline in bloomed flowers and ended when temperatures were steadily below 45^{o}F (approx. September through October) (Núñez 1976). The timing of feeding treatments was similar to when beekeepers feed their hives in this region. Feeding treatments are not the sole source of food that bees consume; feeding managed hives either experimentally or by beekeepers is a supplement to keep the bees from starving in times of low food availability. While bees are feeding on flora outside of experimental control, bees from both treatments have equal access to the same surrounding flora and should not affect analysis of treatment effects.

As part of this experiment, treatment (Supplemental Nectar and Pollen mixture, or 'Nectar,' and Control, 'Sugar'), location of hives, survival status of hives, hive weights, and disease occurrence were all evaluated. Chapter one will focus on hive health measures, such as hive efficiency and growth, disease occurrence of *Nosema* spp. and *Varroa destructor* mites, and survival status of hives throughout the experiment. Chapter two will focus on comparing the above variables to the gut microbiome structure and function to better understand how the supplemental food that honey bees eat, the location of apiaries, the survival status of hives, and hive weights are influenced by or change the gut microbiome.

Hive Weight Analysis

Hive efficiency and growth can be measured by amount of honey produced and population size of colony (Lecocq et al. 2015). Because this is difficult to measure objectively as individual variables, total increase of hive weight over time was substituted as a measure of hive

efficiency and population growth (Meikle and Holst 2015). Between Spring 2018 and Winter 2018, hives were weighed approximately weekly during feeding and monthly between feedings using a digital vertical hanging scale. With weight measurements, honey production, pollen stores, and population growth can be monitored as a function of hive health. To compare hive weight over time and among treatments, locations, and hive survival (hives that survived, hives that were lost in the summer, and hives that were lost in the winter), a linear mixed effect model was used in R studio (version 1.1.463) with Hive ID defined as a random effect to account for repeated sampling. Additionally, each timepoint was analyzed separately using the non-parametric Kruskal-Wallis test in R studio, where hive weight was compared among treatment, survival status, and location. The non-parametric Kruskal-Wallis test was used because the weight data were not normally distributed, even after log transformation. Finally, weight was also compared to gut microbiome structure and function (See Chapter 2).

Disease Susceptibility

Hive disease occurrence was measured through identification and quantification of two common honey bee pathogens: *Nosema* sp. and *Varroa destructor* mites.

Hives were frequently assessed for the fungal microsporidian parasite *Nosema* sp. by visually checking for signs of disease. Because *Nosema* causes dysbiosis, excessive defecation at the entrance or interior of the hive is a sign of *Nosema* infection. This was checked for at each weight and feeding visit to the hive.

Because hives can still have low-level *Nosema* infections without the visual signed, *Nosema* was checked for by visualizing homogenized bee guts under a microscope and counting spores with a hemocytometer (Ellis et al. 2013, Moreira et al. 2012). Samples were collected by using a

soft brush to push bees off of a pulled frame and into a sterile 50 mL collection tube for each hive. Samples were placed on ice in the field and frozen at -80^{0}C upon return to the lab until dissection (Kakumanu et al. 2016). Following a 30 second rinse in 5% bleach solution and two 30 second rinses in sterile water, the whole abdomen of the bee was removed from the head and ground into a paste with 50 mL of distilled water. 50 bees were used in total from each hive analyzed. The paste was then filtered to remove any exoskeleton and centrifuged for 3.5 minutes at 1400 rpm. The liquid was discarded and an additional 50 mL of distilled water was added back into the tube. The liquid and pellet were mixed via pipette and then placed onto the hemocytometer and visualized using a microscope.

For PCR amplification of Nosema, bees were dissected by gently pulling on the stinger under sterile conditions to collect the whole gut following the rinse procedure described above. Five bees were pooled per hive sample to ensure true representative of a typical honey bee gut within the hive (Kakumanu et al. 2016). The whole guts were placed into a single sterile 1.5 mL microcentrifuge tube containing 180 µl lysis buffer and homogenized with a sterile pestle. DNA was extracted using Qiagen DNEasy Blood and Tissue Kit according to the manufacturer's protocol including the lysozyme pre-treatment for Gram-positive bacteria (Walke et al. 2015). DNA was diluted using 200 µl of water and stored at -80^{O}C until PCR amplification. For PCR amplification, general *Nosema* genus primers were used that could identify both *Nosema apis* and *Nosema cerenae* (Chen et al. 2008). Each sample was amplified in triplicate, with each PCR reaction containing 0.5 µl of each forward and reverse primers (10 µM), 5 µl of QuantaBio 5 prime Taq Hotstart Master Mix, 2.6 µl of PCR grade water, 0.4 µl of Magnesium Chloride, and 1 µl of DNA for a total of 10 µl reactions. Water was used in place of DNA template for the

negative controls, which were run for each sample. A positive control containing *Nosema* DNA was also run for each PCR. The PCR reactions were placed in a thermocycler with the following conditions: 1) 94°C for 2 minutes, 2) 94°C for 30 seconds, 3) 55°C for 1 minute, 4) 72°C for 1 minute, 5) 72°C for 5 minutes, and 6) held at 4°C. Steps 2-4 were repeated a total of 35 times. Triplicate reactions for each sample were combined, and amplification was confirmed using 1.5% agarose gel electrophoresis. Samples were considered positive for *Nosema* if there was a band that matched the band of the positive control.

Hives were also monitored for *Varroa* mites once in the fall (September 2018) and once in the Spring (April 2019) using the ethanol roll method, where approximately 100 bees were collected and added to a container with a small sieve (Dietemann et al. 2013). The sieve was placed at the top of the container with the bottom filled with 100% ethanol. The honey bees were placed in the sieve portion and the whole container was shaken for approximately one minute. This coated the bees in ethanol and removed *Varroa* mites, which are small enough to pass through the sieve and collect in the ethanol. Even though the mites are small, they are bright red and easy to visually see and count once in the ethanol. Mite counts were analyzed against treatment and hive location in R using a two-tailed T-test and visualized using a boxplot.

Hive Survival

Hives were monitored during feeding and weighing to ensure than hives were healthy. To determine if a hive was still alive, the following factors were observed: large number of dead bees outside of hive, excessive drone cells in hive indicating a loss of the queen bee, presence of brood cells, and presence of bees within the hive.

Survival rates were analyzed against treatment and location of hive using Fisher's Exact test in R Studio (version 1.1.463).

Results

Hive Weight

While all hives increased in weight over the course of the experiment (linear mixed effect model, all p-values < 0.05), hive weights did not differ over all time between treatments (Figure 1.2; linear mixed effect model, t-value = 0.62, p-value = 0.54), location (Figure 1.3; linear mixed effect model, t-value = -1.45, p-value = 0.17), or survival status (Figure 1.4; linear mixed effect model, hives lost in summer t-value = -1.85, p-value = 0.09, hives lost in winter t-value = 1.62, p-value = 0.13). While it appeared there may be some difference between treatments at particular timepoints or locations based on the graphs, there was no significant difference (Table 1.4).

At three timepoints early in the experiment (June 1st, June 6th, and June 12th), Red Barn hives were significantly heavier than those at Four Lakes (Figure 1.5, Table 1.4). Hive weights were not statistically different among any variables at the microbiome sampling timepoints (all $p > 0.05$, Table 1.4).

Hive weighted differed by survival status later in the experiment on August 23rd (Figure 1.6; Kruskal-Wallis, chi-squared = 7.67, p-value = 0.02), where hives lost in the winter were heavier than hives lost in the summer (Wilcoxon Pairwise Test: p-value = 0.053) but not those that survived (Wilcoxon Pairwise Test: p-value = 0.19). Hives that survived were also not significantly different than those lost over summer (Wilcoxon Pairwise Test: p-value = 0.12).

Disease Susceptibility

Nosema

Nosema infection was not detected via hemocytometer method, nor was it detected via PCR amplification of *Nosema* gene. After analyzing several key winter timepoints, it was determined there was likely little to no *Nosema* infections in either apiary. Additionally, hives showed no major signs of *Nosema* infection in the field. There was no indication of excessive excrement outside the entrance of the hive and, excluding the drone bees expelled from the hive at the beginning of winter, there was not an excessive number of dead bees found at the entrance of hives.

Varroa destructor Mites

There were no significant differences in mite counts between treatments in fall 2018 or the following spring (Fall: Figure 1.7, two-tailed t-test, t-value = 1.83, p-value = 0.13; Spring: Figure 1.8, t-value = -0.75, p-value = 0.59), or between survival status (Figure 1.9, two-tailed t-test, t-value = 1.55, p-value = 0.2). Location of hives was not evaluated as a variable of mite infestation because during fall sampling, only Four Lakes hives were observed and during the spring sampling, Red Barn had one remaining hive, limiting sample size for statistical analysis.

Hive Survival

Hives were found to be dead at two separate timepoints: Once in August 2018 (referred to as Dead Summer, or “DeadS”) and once in March 2019 (referred to as Dead Winter, or “DeadW”). Six hives at Red Barn and one hive at Four Lakes were lost in August. One hive at Red Barn and four hives at Four Lakes were lost over winter (Table 1.5). There were no significant differences in survival between treatments (Fisher’s exact, p-value = 1). There was a

trend in which Red Barn had slightly higher hive loss during summer (Fisher's exact, p-value = 0.059).

Discussion

Hive Weight

While all hives increased in weight over the course of the experiment as they became established, feeding honey bees with a nutritional supplement mimicking natural pollen and nectar did not affect growth of hive population or honey stores, as measured by hive weight. This is in contrast with other published studies; for example, one study examined the effect of an assortment of sugar diets (e.g. sucrose, glucose, and fructose) on hive health factors and showed that hives fed the sucrose diet gained the most weight out of their treatment groups (Guler et al. 2018). Other factors that were not measured or controlled for as part of this field experiment could have a stronger effect on hive growth, such as water availability, external food sources, weather patterns, predators, and pathogens. For example, the less time honey bees have to dedicate to collecting water to bring into the hive to cool by evaporation, the more time and resources they can dedicate to foraging for nectar and pollen; therefore, if there is a close water resource, hives can product more honey (Le Conte and Navajas 2008). Additionally, honey bees were not limited to the nutrient supplementation provided; the nutrient supplementation was not meant to replace every nutrient the bees need but rather enhance natural resources. Therefore, the honey bees were still able to forage for nectar and pollen. It is challenging to measure what food resources the honey bees are bringing back into the hive and quality of food resources is important for many factors of hive health, including brood and honey production (Maes et al. 2016). One study in particular found that

supplementing hives with pollen increased brood production in young queens during the fall and early spring (Ricigliano et al. 2018). Because the queen bees used for this experiment were young queens (less than a year old), I would have expected this pattern to be present in my experimental hives as well. Weather patterns also influence foraging for food and water; drought can cause a halt in nectar flow as well as an increase in predatory insects like yellow jackets (Navajas 2008). These environmental factors could have had a stronger influence on hive growth than the nectar supplementation tested here.

Hives lost over winter were significantly heavier than hives lost over summer or those that survived the experiment. However, this pattern was only observed at a single timepoint at the end of August. This is likely due to two factors: first, this weight timepoint was one week prior to observing the loss of seven hives in summer; these hives were likely already losing a significant amount of population and honey stores (see survival discussion below). Second, hives that grow rapidly and are very heavy in summer, such as those that were lost over winter in this study, are subject to swarming (Rangel & Seeley 2012). Swarming events are where hive populations grow to exceed the size of the hive boxes and the hive splits its population, taking up to 75% of the bee population and the old queen to find a new home to populate. This can weaken a hive if swarming events occur just before winter (Rangel & Seeley 2012).

Location of hives is important for hive growth and production; the nutrient and water resources available within five miles of the hive impact honey production and brood numbers (Guzman et al. 2019). Hives at Red Barn were significantly heavier than the Four Lake hives at three timepoints during late spring (June 1st through June 12th). These hives started out strong with large population and honey stores and rapid growth, which led to some hives swarming

during early to mid-summer. This likely explains why I observed the initial hive weight differences, with Red Barn hives being heavier pre-swarm. It is possible the hives at Red Barn had better resources, such as external pollen sources and water sources in Spring and early summer than at Four Lakes; this would account for the difference in initial population growth and honey stores. Although it is difficult to account for the many confounding factors in a field study and to identify mechanisms explaining the observed patterns, my experiment was able to determine that hive weight was related to timepoint, apiary location, and survival status, but not my supplemental diet treatment.

Disease Susceptibility

Nutrition plays an important role in a hive's susceptibility to *Varroa* mite infestation. For example, previous studies have shown that the landscape around apiaries and therefore the floral resources available for foraging significantly influences *Varroa* mite infestation (Dolezal et al. 2016, Giacobino et al. 2017). Additionally, *Varroa* mite infestation has been shown to decrease host metabolic pathways, specifically protein metabolism, and that this is not easily reversible once a hive becomes infested with *Varroa* mites (Alaux et al. 2011). However, it could be that supplementing with protein prior to infestation, as I did with my nutrient supplementation mixture provides honey bees with the protein they need to continue to grow larvae and mitigate this effect of *Varroa* mite to allow honey bees to resist infestation. This could explain why there was no significant difference between treatment or survival status on infestation of *Varroa destructor* mites. Alternatively, the mite count numbers in fall were relatively low (0-4 mites per 100 bees) and likely not a strong selective pressure in either apiary, possibly due to the sugar-water feeding in the control hives; previous research has shown that

hives fed a sugar-water supplementation had an augmented production of host antimicrobial peptides, increased metabolic pathways, and increase in genes affecting honey bee longevity (Alaux et al. 2011). Therefore, it is possible that the sugar-water alone was enough to mitigate *Varroa* infestation. It is difficult to determine an effect of treatment on mite infestation with such low mite infestation.

Although the fungal gut pathogen *Nosema* was not detected at either apiary, we only tested for this pathogen using molecular methods in select fall and winter timepoints because this is when *Nosema* is typically most prevalent (Webster et al. 2004). It could be that *Nosema* is prevalent at different times of the year in this region than in better studied areas. Both species of *Nosema* (*N. ceranae* and *N. apis*) that infect honey bees appear to be widespread throughout the United States, but there is little research to support the distribution and prevalence of *Nosema* specifically in the Pacific Northwest (Grupe and Quandt 2020). More research across seasons and geographical locations is needed to fully understand the scope of *Nosema* infection in this region.

Survival

Hive loss annually is unfortunately a common occurrence for beekeepers. During 2015-2016, annual hive loss in the United States was estimated at 40.5% of managed hives, with winter losses at 26.9% and summer losses at 23.6% (Kulhanek et al. 2017). In the short term, hive losses fluctuate; this is thought to be due to fluctuating environmental conditions, such as weather between different years (Potts et al. 2010). However, there is a long-term trend of increasing honey bee hive losses (Seitz et al. 2016).

Washington hives were more impacted than the United States average. Washington State is in the top ten states for most managed honey bee hives (Bee Informed Partnership,

2021). Hives in Washington state are essential for the agricultural production of apples, cherries, pears, and raspberries (Klein et al. 2007, USDA 2021). According to the Bee Informed Partnership, Washington hive loss was at 62.1% for the winter season, summer loss at 18.5%, and an annual average of 68.6% loss for the 2018/2019 season (Bee Informed Partnership 2021). Hive losses can vary between specific geographical locations and hive management practices. For example, Western Washington sees higher precipitation rates than Eastern Washington, which may contribute to a higher hive loss in Eastern Washington due to longer periods of draught (Wise 2010). Hive management is also a critical part of hive survival. Placement of hives within an apiary, overwintering practices, and disease management all impact hive survival (Oberreiter & Brodschneider 2020).

Hive loss between our two apiaries exceeded this average in the summer season and annually; in summary, 43.75% of hives were lost over summer and 55.56% of hives were lost over winter, for a total of 75% hive loss. Six of the seven hives lost over the summer were at the Red Barn apiary. As mentioned above, this is likely due to the large amount of swarming activity in that apiary in early summer. In late summer, this location had a large infestation of yellow jackets (*Vespula* sp.) predators; the hives were found nearly empty of honey bees with yellow jackets robbing whatever honey stores were left. Yellow jackets are known predators of honey bees, robbing honey stores when other nutrient sources are depleted (Pusceddu et al. 2018). The swarming activity around this timepoint likely left these hives with too small of a population to defend their hive from the yellow jackets. Due to the large number of hives lost over summer, Red Barn had slightly higher hive loss than Four Lakes.

Over winter, hives that were heavier in the fall months were the ones that were lost. There could be two possible reasons for this: first, there could have been swarming activity in the fall that weakened the hives, or second, they had too large of a population to sustain over winter with the honey stores they had prepared for winter. Food storage over winter is vital for hive survival; honey bees need to consume honey over the winter for the energy required to keep their hive warm and, with no plants available for forage for nectar during the winter, it is important that bees have enough food storage to last through the winter months (Wright et al. 2018).

Treatment did not have an effect on hive survival. This is not what I expected because nutrient supplementation can increase immunocompetence, brood and honey production, and metabolism of nutrients (Alaux et al. 2010, Dolezal & Toth 2018, Guler et al. 2008, Ricigliano et al. 2018). It is possible this mixture of nutrients did not contain everything needed to boost honey bee health, particularly regarding the protein content. The sole protein included in my supplement was casein; pollen protein composition is much more complex and diverse, and other nutrient studies generally include actual pollen samples for the protein content instead of isolated protein (Roulston et al. 2000, Tristchler et al. 2017). Future studies should include a wider range of protein available after potentially analyzing pollen protein sources locally to determine what honey bees in the area are consuming.

CHAPTER 2: EFFECTS OF NUTRITION ON HONEY BEE GUT BACTERIAL COMMUNITY STRUCTURE & FUNCTION

Methods

For chapter two, I hypothesized that supplementing hive colonies with a mixture of sugar, water, and a select group of amino acids and vitamins that mimic naturally occurring pollen and nectar would have a beneficial effect on the structure and function of the gut microbiome of honey bees.

Honey bee samples described in this chapter are referring to the experiment and hive set up described in chapter one. For experimental design and set up, see chapter one.

Sampling & Sequence Prep

To characterize gut bacterial communities, honey bees were sampled from a brood frame to get a mixture of young and old bees before and after each cycle of supplemental feeding (Table 3). Samples were collected by using a soft brush to push bees off of a pulled frame and into a sterile 50 mL collection tube for each hive. Samples were placed on ice in the field and frozen at -80^0C upon return to the lab until dissection (Kakumanu et al. 2016). Following a 30 second rinse in 5% bleach solution and two 30 second rinses in sterile water, bees were dissected by gently pulling on the stinger under sterile conditions to collect the whole gut. Five bees were pooled per hive sample to ensure true representative of a typical honey bee gut within the hive (Kakumanu et al. 2016). The whole guts were placed into a single sterile 1.5 mL microcentrifuge tube containing 180 µl lysis buffer and homogenized with a sterile pestle. DNA was extracted using Qiagen DNEasy Blood and Tissue Kit according to the manufacturer's protocol including

the lysozyme pre-treatment for Gram-positive bacteria (Walke et al. 2015). DNA was eluted from collection filter using 200 µl of water and stored at -80^{O}C until PCR amplification.

The V4-V5 regions of the 16S rRNA gene were amplified with PCR using barcoded-515F and 926R primers (primer sequences without linker, pad, barcode, or Illumina adaptor: forward sequence: GTGYCAGCMGCCGCGGTAA, reverse sequence: CCGYCAATTYMTTTRAGTTT) following the 16S Illumina amplicon protocol from the Earth Microbiome Project (EMP) (Walters et al. 2016, Parada et al. 2016). These primers were selected because they amplify the portion of the 16S rRNA gene in bacteria that has conserved regions for the primers to bind, but also hyper-variable regions within the amplified fragments to identify different types of bacteria in the community. The amplicons were also barcoded on the forward primer so that samples may be tagged with unique barcode sequences per sample and multiplexed for sequencing process. Following sequencing, samples can be demultiplexed and identified using the barcode in the bioinformatic data analysis steps.

Each sample was amplified in triplicate, with each PCR reaction containing 2µl of each forward and reverse primers (10 µM), 10 µl of QuantaBio 5 prime Taq Hotstart Master Mix, 12 µl of PCR grade water, and 2 µl of DNA template diluted with molecular water 1:10 for a total of 25 µl reactions. Water was used in place of DNA template for the negative controls, which were run for each sample. The PCR reactions were placed in a thermocycler with the following conditions: 1) 94^{o}C for 3 minutes, 2) 94^{o}C for 45 seconds, 3) 50^{o}C for 1 minute, 4) 72^{o}C for 1.5 minutes, 5) 72^{o}C for 10 minutes, and 6) held at 4^{o}C. Steps 2-4 were repeated a total of 35 times. Triplicate reactions for each sample were combined, and amplification was confirmed using 1.5% agarose gel electrophoresis. DNA concentration of each sample was measured using a

Qubit 4.0 fluorometer with the dsDNA High Sensitivity assay kit, and samples were pooled in equimolar concentrations into a sterile 1.5 mL tube. Finally, the pooled sample was cleaned using the Qiagen QIAquick PCR Clean Up Kit. The final concentration of the pooled, cleaned sample was measured with the Qubit fluorometer at 42.1 ng/µl. To characterize the structure of the gut bacterial community, DNA was sequenced in January 2019 by Dana-Farber Cancer Institute at Harvard University using a 250 base pairs (bp) paired-end approach on the Illumina MiSeq platform (Caporaso et al. 2011).

Importing Data and Initial Filtering

DNA sequence data was processed with the bioinformatics program Quantitative Insights into Microbial Ecology 2 (MacQIIME v2-2019.1) for gut microbiome community structure analysis (Bolyen et al. 2020). Data was imported into QIIME2 using the import plugin with a manifest file and type parameter "SampleData[PairedEndSequncesWithQuality]". Initial filtering steps included QIIME plugin DADA2 (Callahan et al. 2016) and filtering of mitochondria, chloroplast, and unassigned sequences. DADA2 was used for quality control of sequencing data because it is customizable for where to trim reads on either end based on quality scores and is the only quality filtering plugin available with QIIME2 that supports paired end sequences. Trim location of read was chosen based off of mean quality score of each base pair (Figure 2.1). Forward reads were trimmed at 12 base pairs at 5' end and 228 base pairs at 3' end. Reverse reads were trimmed at 13 base pairs at 5' end and 217 base pairs at 3' end.

After initial filtering steps, number of reads in each sample sequences were analyzed. One sample (nectar replicate from Four Lakes in September timepoint) was removed from the study due to extremely low sequence counts; the sample amplified only two sequences.

Sequence counts per sample ranged from 30,834 to 125,576, with a total sequence count of 3,438,513 and a mean of 61,971.5 sequences per sample. Across all samples, these sequences were clustered into 339 unique operational taxonomic units (OTUs) based on 100% sequence similarity. Samples were then visualized via alpha rarefaction plugin, which graphically shows the number of sequences needed to accurately capture bacterial diversity within each sample (Figure 2.2). Based on this, the data were rarefied at 30,834 sequences per sample to standardize the sequencing depth (and thus the sampling effort) per sample. This value was chosen to capture the maximum amount of diversity without losing any further samples.

Phylogenetic Tree

The phylogenetic tree was made using align-to-tree-mafft-fasttree QIIME plugin (Katoh & Standley 2013). This plugin creates a phylogenetic tree by first aligning sequences using MAFFT and masking any phylogenetic uninformative or unassigned sequences. From there it creates a tree by inferring a midpoint based on the longest tip-to-distance unrooted tree. For further analysis, the masked, rooted tree from this plugin was used.

Taxonomy

Taxonomy was assigned using a custom classifier trained for 515F-926R primers using the Silva 16S database v132 99% OTUs reference sequences pre-processed by QIIME to remove any ambiguous sequences, replicated taxonomy, or errors (Glöckner et al. 2017). Reads for the 515F-926R primer set were extracted using feature-classifier extract-reads QIIME plugin and classified using the classify-sklearn naïve Bayes taxonomy classifier plugin (Bokulich et al. 2018).

After all filtering was complete, the diversity core-metrics-phylogenetic plugin was used to calculate four alpha diversity metrics (observed OTUs, Shannon, Evenness, Faith's

Phylogenetic diversity) and four beta diversity metrics (Bray Curtis, Jaccard, Unweighted Unifrac, Weighted Unifrac). Because the following statistics used for both alpha and beta diversity do not take into consideration repeat sampling of hives over time, samples were analyzed per timepoint separately.

Alpha Diversity

Alpha diversity is the measure of diversity within a particular ecosystem, in this case the honey bee gut microbiome, measured by the number or abundance of each species. The QIIME core-metrics-phylogeny plugin results in four different alpha diversity metrics, allowing a range of quantitative and qualitative analyses. Observed OTUs measures the number of distinct OTUs within a sample, defined as richness. Pielou's evenness measures the relative abundance of each OTU, defined as evenness. Shannon diversity metric measures both the richness and evenness. Finally, Faith's phylogenetic diversity measures richness and abundance with consideration for the phylogenetic relatedness of each OTU.

To analyze the effects of treatment, location, survival status, and hive weight on each of these four alpha diversity metrics, the diversity alpha-group-significance plugin was used to analyze categorical data (Treatment, Location, Status) and the diversity alpha-correlation plugin was used to analyze continuous data (Weight). The alpha-group-significance plugin uses a Kruskal-Wallis test to test for differences in alpha diversity among variables and is visualized with box plots, while the alpha-correlation plugin utilizes Spearman rank test and is visualized using scatter plots.

Beta Diversity

Beta diversity is a measure of species diversity between ecosystems, testing differences in microbiome composition and structure between variables. The QIIME diversity core-metrics-phylogeny plugin results in four beta diversity metrics: Bray-Curtis, Jaccard, Weighted Unifrac, and Unweighted Unifrac. Jaccard and Unweighted Unifrac compare OTUs between two groups based on presence/absence of species, while Bray Curtis and Weighted Unifrac compare both presence/absence and abundance of OTUs in each ecosystem, or bee gut sample. The weighted and unweighted Unifrac metrics both additionally consider phylogeny of OTUs. Utilizing all four beta diversity metrics is vital for observing if significant changes in bacterial diversity between communities are driven by simple presence/absence of a bacterial group or if this change is driven by relative abundance and relatedness of these bacterial groups as well.

To analyze these four beta diversity metrics, I used a permutational multivariate analysis of variance (PERMANOVA) on each distance or similarity matrix via the diversity beta-group-significance plugin in QIIME to test for differences in microbiome structure between treatments, location of apiaries, and survival status. To test for a correlation between hive weight and microbiome structure, I used a Mantel correlation test on each distance/similarity matrix via the diversity beta-correlation plugin in QIIME. Both tests were visualized using a principal coordinate analysis (PCoA) using QIIME emperor plot function.

Relative Abundance of Bacterial Groups

To test for differences in relative abundances of bacterial genera between variables, Linear Discriminant Analysis Effect Size (LEfSe) was used (Segata et al. 2011). This application compares the relative abundance of OTUs between variable groups, determining if an OTU is higher abundance or unique to a particular group by first running the data set with a non-

parametric Kruskal-Wallis test to determine bacterial groups with significantly different relative abundances by variable of interest. Any significant bacterial groups are then analyzed using a linear discriminant analysis (LDA) to determine the effect size of each group (Segata et al. 2011). Additionally, relative abundance was visualized with taxa bar plot QIIME plugin.

Bacterial Community Function

To infer what kind of functionality the bacterial community may provide to the honey bee gut, and how this potential function varies by treatment, the QIIME plugin Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUSt) was used. This approach identifies theoretical community function analysis based off predictive metagenomic profiling of the 16s rRNA gene (Langille et al. 2013).

The program Statistical Analysis of Metagenomic Profiles (STAMP) was used to analyze the PICRUSt output data (Parks et al. 2014). This program analyzes the relative abundances of predicted genes between variables using the non-parametric statistical test Kruskal-Wallis with Bonferroni p-value correction as well as a Games-Howell post-hoc test for multiple group variables and White's non-parametric t-test for two group variables with a bootstrap confidence interval.

These tools will help identify how bacterial communities differ in composition and function between treatments to better understand which bacterial groups may correlate with nutrient uptake, hive efficiency, and survival under stress.

Results

Alpha Diversity

Treatment (Nectar vs Sugar), Location

There were no significant differences at any timepoint when comparing bacterial communities between the two treatments (Nectar and Sugar) or hive location across all four alpha diversity metrics (Table 2.1, all $p > 0.05$).

Hive Weight

Bacterial diversity was significantly negatively correlated with both hive weight (observed OTUs, Figure 2.3, $r_s = -0.93$, p-value = 0.007 and Shannon, Figure 2.4, $r_s = -0.83$, p-value = 0.004) and percent weight gain (observed OTUs, Figure 2.5, $r_s = -0.99$, p-value = 0.0003) at the September timepoint, where heavier hives had lower bacterial community diversity than lighter hives. There were no correlations between weight or weight gain and bacterial diversity at the May, April, August, or October timepoints (Table 2.1, all $p > 0.05$).

Status (Alive vs Dead Winter vs Dead Summer)

During May, hives that died later in the summer had significantly lower diversity than those that survived the experiment (observed OTUs, H = 4.03, p-value = 0.045) and those that were lost over winter (observed OTUs, Figure 2.6, H = 6.90, p-value = 0.0086). Hives lost over winter and hives that survived were not significantly different (observed OTUS, Kruskal-Wallis, H = 0.009, p-value = 0.92). Similarly, at the October timepoint, hives lost over winter had significantly lower diversity that hives that survived the winter (Faith's phylogenetic diversity, Figure 2.7, H = 6, p-value = 0.014). At the timepoints in April, August, and September, there were no differences among survival status for the four alpha diversity metrics (Table 2.1, all $p > 0.05$).

Beta Diversity

Treatment

Treatment did not influence the bacterial community structure across all four beta diversity metrics across all timepoints (Figure 2.8, PERMANOVA, all $p > 0.05$).

Location

Bacterial community structure did not differ between apiary locations at the April or May timepoints (Figure 2.9, PERMANOVA, all $p > 0.05$); September timepoint was not analyzed as only Four Lake hives were sampled, and August and October timepoints were excluded due to the fact there were only two hives remaining at Red Barn at those timepoints.

Weight

Hive weight did not correlate with microbiome composition at any of the five timepoints (Table 2.2, Mantel Correlation Test, all $p > 0.05$).

Status

Survival status of hives had significantly different microbiomes at several timepoints. Interestingly, in April at the start of the experiment, all three survival statuses had significantly different microbiome structures (Bray-Curtis, Figure 2.10, pseudo-F = 1.98, p-value = 0.025). Although not significant, hives that survived tended to be more similar to those lost in winter (Bray-Curtis, pseudo-F = 1.9, p-value = 0.095) than those lost in summer (Bray-Curtis, pseudo-F = 2.08, p-value = 0.073) when compared pairwise. Hives lost over winter differed most from hives lost over summer (Bray-Curtis, pseudo-F = 1.94, p-value = 0.033).

There was a similar pattern at the May timepoint. Hive survival status had significantly different microbiome composition overall (Jaccard, Figure 2.11, pseudo-F = 1.34, p-value = 0.047). Pairwise, there were no significant differences or potential trends, except again when comparing hives lost over summer to hives lost over winter (Jaccard, pseudo-F = 1.43, p-value = 0.063).

In August, hives that survived and those that were lost over winter had statistically different microbiome compositions (Jaccard, Figure 2.12, pseudo-F = 1.4, p-value = 0.044). This microbiome sampling was taken after hives had died over summer. There were no significant differences at the September timepoint in regard to survival status. Similar to the August timepoint, in October leading up to the winter, hives lost over winter had significantly different microbiome composition than hives that survived (Unweighted Unifrac, Figure 2.13, pseudo-F = 2.54, p-value = 0.023).

Relative Abundance by Bacterial Genus

Overall, there were 339 unique bacterial groups (100% OTUs) found across all samples. The genus *Lactobacillus* was the most abundant across all samples, ranging from 93% to 41% relative abundance with a mean relative abundance of 71%. *Gilliamella* (mean 12%), *Snodgrasella* (mean 11%), *Commensalibacter* (mean 2%), *Bifidobacterium* (mean 1%), *Bartonella* (mean 1%), and *Frischella* (mean 1%) were also present in all samples in relatively high abundance (Figure 2.14).

Treatment

At the May timepoint, *Arsenophonus* was significantly higher in Nectar treated hives than Sugar treated hives (Figure 2.15, Nectar Mean = 2.6%, Sugar Mean = 0%, LDA = 4.45, p-value = 0.011). In August, *Commensalibacter* was significantly higher in Sugar hives, although it was in low relative abundance overall in both treatments (Figure 2.16, Nectar Mean = 0.15%, Sugar Mean = 0.54%, LDA = 3.66, p-value 0.027). Treatment had no effect on relative abundance of bacterial groups at the April, September, or October timepoints (all $p > 0.05$).

Location

At the August timepoint, three bacterial genera had higher relative abundances at Red Barn than Four lakes: *Gilliamella* (Figure 2.17, Four Lakes mean = 12.8%, Red Barn mean = 20.7%, LDA = 4.66, p-value = 0.04), *Snodgrasella* (Figure 2.18, Four Lakes mean = 7.7%, Red Barn mean = 18.2%, LDA = 4.78, p-value = 0.04), and *Pseudomonas* (Figure 2.19, Four Lakes mean = 0%, Red Barn mean = 0.43%, LDA = 3.97, p-value = 0.005); additionally, *Lactobacillus* was higher at Four Lakes than Red Barn at this timepoint (Figure 2.20, Four Lakes mean = 75%, Red Barn mean = 52%, LDA = 5.05, p-value = 0.04).

In October, there were two genera that were higher in relative abundances at Red Barn than Four Lakes: *Arsenophonus* (Figure 2.21, Four Lakes mean = 0%, Red Barn mean = 0.017%, LDA = 4.85, p-value = 0.005) and *Bartonella* (Figure 2.22, Four Lakes mean = 1.3%, Red Barn mean = 15%, LDA = 4.85, p-value = 0.04).

There were no differences in bacterial relative abundances between locations at the April and May timepoints (all p-values > 0.05). The September timepoint was not included as there was only one location sampled.

Status

Interestingly, at the April timepoint, *Snodgrasella* was higher in hives that survived the experiment than those that died over summer or over winter (Figure 2.23, Alive mean = 12.5%, Dead Summer mean = 5.7%, Dead Winter mean = 7.8%, LDA = 4.81, p-value = 0.043).

In September, *Providencia* was higher in hives that survived the experiment than those lost over winter, although its relative abundance was very low (Figure 2.24, Alive mean = 0.02%, Dead Winter mean = 0%, LDA = 2.35, p-value = 0.049).

<u>Community Function</u>

In total, there were 263 predicted functions observed across all samples. There were no functions significantly associated with treatment, location, or survival status across all timepoints (all p-values > 0.05).

Discussion

Gut Microbiome Structure

The most abundant bacterial groups across all samples were *Lactobacillus*, *Gilliamella*, *Snodgrasella*, *Bifidobacterium*, and *Commensalibacter*. This group of genera, outside of *Commensalibacter*, is commonly found as the core group in honey bee gut microbiome (Kwong & Moran 2016). *Commensalibacter* is usually not a genus associated with the core honey bee gut microbiome, meaning that it is not a member of the eight bacterial groups always present in the gut regardless of time of year or diet, but is still commonly found in the gut and generally fluctuates throughout seasons (Kwong & Moran 2016).

Treatment

The nectar supplementation treatment had no significant effects on overall gut microbiome structure. However, when examining the relative abundances of particular bacterial taxa, there were patterns of differential abundances between treatments. In May, there was a higher abundance of the bacterium *Arsenophonus* in the nectar-treated hives, although this genus was still in low abundance overall in the community (2.6% mean in nectar hives and 0% mean in sugar hives). Interestingly, this bacterium is thought to be a pathogen transmitted by *Varroa destructor* mites (Yanez et al. 2016). *Arsenophonus* has been found to be more abundant in hives with symptoms of colony collapse disorder; this may be due to the correlation with *Varroa destructor* mites (Yanez et al. 2016). Seasonally, *Arsenophonus* is

typically lower in spring and fall and peaks in abundance in summer, following seasonality of *Varroa* mites (Drew et al. 2021). Unfortunately, we were unable to evaluate the relationship between the abundance of this bacterium and *Varroa* infestation since we did not take mite counts at this timepoint. While it is unreasonable to assess this relationship without *Varroa* mite counts, this could suggest an increased number of *Varroa* mites in nectar treated hives at this timepoint.

In August, *Commensalibacter* was significantly higher in sugar-treated hives than nectar-treated hives. *Commensalibacter* is a genus of acetic acid bacteria (AAB) that ferment sugars; as such, this bacterial group is reasonably more abundant with more sugar present (Crotti et al. 2010). However, with sugar also a main ingredient in the nectar treatment, I would expect there to be no difference between treatments. Potentially, this bacterium was limited by other bacteria that could metabolize the additional nutrients present.

Other studies that have evaluated the impact of nutrition on gut microbiome composition have found that members of the core microbiome differ between supplementation and control. A study by Rothman et al. found that *Gilliamella*, *Lactobacillus*, and *Bartonella* decreased with supplemental forage in comparison to the control (Rothman et al. 2018). This is interesting because all three are associated with metabolizing key nutrients of honey bee diet such as carbohydrates and degradation of pollen. Another study evaluated the influence of syrup mixtures against honey as a nutritional supplementation and found an increase in *Rhizobiales* (the order that contains *Bartonella apis*) and *Bifidobacteria* in honey and wheat starch syrup treatments in comparison to standard sugar water (D'Alvise et al. 2018). I would have expected a similar pattern with my experiment because there are more nutrients

available to digest in my nutrient supplementation, bacteria that support the metabolism of nutrients such as *Gilliamella*, *Snodgrasella*, and *Lactobacillus* would be increased with nutrient supplementation (Kwong and Moran 2016, Lee et al. 2018, Lee et al. 2015a, Zheng et al. 2016, Kwong et al. 2017).

For future studies, the nutrient composition of supplemental feed should be further evaluated in a controlled setting such as a cage experiment with nutrients broken down into smaller categories. This would allow for a more precise evaluation of which nutrients fortify honey bee health best before nutrients are combined into a single supplementation and provided to whole honey bee hives.

Location

For the first four months of the experiment, the gut microbiomes of bees at the two apiaries did not differ. In August and October, there were several bacterial groups that differed in relative abundance between the two apiary locations. However, there was a low sample size at these two timepoints because there were only two hives remaining at Red Barn. While this limits the validity of the statistics, there was a large difference in relative abundances in bacterial groups between locations that it merits mentioning. Additionally, the gut microbiome is known to differ spatially; this is likely due to the landscape around the hives and as such where the honey bees are foraging for food and water (Donkersley et al. 2018). Thus, it is expected that the gut microbiome will differ between apiary locations.

Survival Status

Interestingly, hive survival was correlated with several aspects of the honey bee gut microbiome. First, hives that survived the experiment had significantly higher bacterial diversity than those that did not survive, at the sampling timepoint before they were discovered dead,

both in May before summer hives were discovered dead and in October before winter hives were discovered dead. This could mean that a more diverse gut microbiome has some benefit that allows honey bees to survive stressors. A diverse gut microbiome is important for honey bee health because each bacterium has a function to contribute to factors of health such as metabolic activity and immune response; these bacteria work together to create a working ecosystem of symbiotic functions that assist with host health (Kesnerova et al. 2017). This pattern is also seen in human gut microbiome and is associated with a diverse diet (Heiman & Greenway 2016). Additionally, a diverse skin microbiome has been found to decrease disease intensity and prevalence by pathogenic fungi in amphibians (Walke et al. 2017), trout (Lowrey et al. 2015), and bats (Lemieux-Labonte et al. 2017); this pattern could also apply to pathogens that infect the honey bee gut such as the microsporidian fungi *Nosema*.

In addition, survival status of hives was correlated with different microbiome communities at several timepoints (April, May, August, and October). Interestingly, this "survival microbiome biomarker" was detected as soon as the first sampling timepoint in April, taken before hives were introduced into their new homes. However, it is important to note that this distinct gut microbiome structure between survival status groups was not significant across the same beta diversity metrics over time. In April, the Bray-Curtis metric detected these differences, while the Jaccard metric detected differences in May and August, and, finally, the Unweighted Unifrac metric detected differences in October. This suggests that in the beginning of the experiment, when hives arrived from the initial single apiary, the actual composition was similar, but the relative abundance of particular bacterial groups, such as *Snodgrasella*, differed and was an important distinguishing factor for hive health. Later in the experiment, the

composition shifted, indicated by Jaccard and unweighted UniFrac being different, with members of the community either being lost or gained, and this had an impact on ultimate hive survival.

Interestingly, *Snodgrasella* had higher relative abundance in April in hives that ended up surviving the experiment. *Snodgrasella* is recognized as a core honey bee gut microbe and is involved with downstream metabolism of sugar byproducts such as carboxylates (Moran 2015). Additionally, *Snodgrasella* is associated with higher survival rates when exposed *in vitro* to the pathogen *Escherichia coli* (Kwong et al. 2017). It is difficult to say what kind of influence this bacterium had over hive health in regard to overwintering success at such an early timepoint, but the initial increased abundance could have influenced community composition changes throughout the entire experiment and influenced survival indirectly. In fact, *Snodgrasella* is one of the first gut microbes to colonize bee guts, forming a biofilm on the gut lining and modifying the gut environmental conditions for later colonizing microbes (Sauers & Sadd 2019). Thus, the relative abundance of this key early colonizer can have long term implications on gut community structure and hive survival, as seen in my study. Future research should focus on manipulating the microbiome by adding and removing this microbe from the gut community to further evaluate its impacts on bee and hive health and survival.

As hives became established in their new homes during the spring and summer months, whole bacterial groups could have been added or removed, causing Jaccard to pick up on differences solely in presence/absence. In particular, although at very low relative abundances (0-0.02%), *Providencia* was found in higher abundances in hives that survived than those that were lost over winter at the September timepoint. *Providencia* is not well studied in honey

bees; first identified in the honey bee gut in a study published in 2019, its function is not well understood (Khan et al. 2017, Sladjana et al. 2019). In other insects such as *Drosophila*, this bacterium is typically pathogenic, which is not what I would expect from a bacterium correlated with hive survival (Sladjana et al. 2019). However, with such low relative abundance found here, this bacterium likely did not have much influence on hive survival.

While changes were detected in overall gut microbiome structure, no particular bacterial groups were significantly different in relative abundances between survival statues in October. At this point, the hives had been established for six months; it is possible the Unweighted Unifrac was detecting changes in strain level variation of bacterial groups, since this metric measures differences in phylogenetic relatedness among microbial community members.

Future research could focus on removal and inoculation of honey bee guts with *Snodgrasella* (such as Kwong et al. 2017) and *Providencia* to further elucidate their role in hive health and survival. *Snodgrasella* plays a major role in nutrient digestion (Moran 2015) but is not commonly found in probiotic mixtures (e.g. Super DFM Commercial Probiotic). *Providencia* is not well understood, so evaluating this bacterium's influence on hive health, especially in low abundances as seen in my study, would be a novel contribution to the field. Further identifying the role of overall diversity of the gut microbiome of honey bees would also help us understand how the composition of the gut microbiome impacts hive health. Furthermore, it would be useful to evaluate the macroecological theory of the diversity-function relationship (Carroll et al. 2011) in a microbial system like the honey bee gut microbiome to expand the generalizability of this theory and prove its applicability on additional ecological systems.

Weight

September was the only timepoint when weight correlated negatively with microbiome differences. Hives that were heavier in weight had less bacterial diversity than lighter hives. This could mean that increased bacterial diversity correlates with less brood and honey production in hives; it's possible that with additional groups of bacteria, the bacterial groups needed to assist with nutrient digest were in lower abundance, especially in a gut microbiome ecosystem that is relatively simple in comparison to other organisms. For example, the human gut microbiome is much more complex but there is some redundancy and overlap between bacterial group commonly found in the gut and their role in nutrient digestion (Vieira-Silva et al. 2016). While brood and honey productivity have been studied in relation to nutrient supplementation, the composition of the gut microbiome and hive productivity have not been closely researched; this would be a good area for future studies (Ricigliano et al. 2018).

Gut Microbiome Function

There were no functions significantly correlated with treatment, location, or status. It is possible that these three factors did not influence the gut microbiome in a way that altered the overall predicted function of the ecosystem. PICRUSt has been used successfully to study predicted function shifts in the honey bee gut microbiome in other studies, such as the study by Kakumanu et al. that examined the influence of pesticide exposure on the honey bee gut microbiome structure and function and found that genes associated with oxidative phosphorylation increased and sugar metabolism decreased with pesticide exposure (Kakumanu et al. 2016). Another study examined how the gut microbiome structure and function differed between social statuses of honey bees, finding several significant differences between nurse and foraging bees (Yun et al. 2018). Additionally, PICRUSt is frequently used to

analyze predicted function in human gut microbiome studies (Baxter et al. 2014, Heinsen et al. 2016, Zilberman-Schapira et al. 2016).

Because the shift in the gut microbiome composition might have been too subtle to detect microbial functional differences using PICRUSt, future studies should include full metabolomic analyses, focusing on metabolites known to be present in the honey bee gut microbiome that impact host nutrition metabolism (i.e. carbohydrate metabolism and pectin degradation) and support host immune defenses (i.e. antimicrobial peptides) (Engel et al. 2012, Kesnerova et al. 2017, Kwong et al. 2017).

CONCLUSIONS

While the original goal of identifying a beneficial sugar treatment supplemented with protein and micronutrients did not provide any promising solutions for beekeepers, it is very interesting that survival status correlated with microbiome structure, especially at timepoints so early on in the experiment. With increased sample size and additional apiary locations to observe if this pattern is repeatable, gut microbiome sequencing could be a potential tool to add to the modern hive health observation techniques. Biomarkers in the gut microbiome to identify if a hive will be successful would be useful on the industrial scale as well as provide information as to which bacteria are key to hive health and success. Future studies could focus on identifying these specific "survival" bacterial groups and testing a probiotic mixture to observe if altering honey bee gut microbiome to a "healthy" biomarker can achieve hive success and survival. Current probiotic mixtures are supplementing with important members, like the lactic acid bacteria (mainly *Lactobacillus*); it is possible that honey bees could benefit from a wider range of bacterial groups that have more functionality within the gut.

Additionally, in future studies, it would be interesting to break down the protein and micronutrient mixture in a laboratory setting with caged bees to identify what nutrients and in what quantities help honey bee health. This would remove the confounding factors of the field to be able to identify what nutrients are vital before repeating this study in the field. Nutrient stress is becoming an increasingly large stressor in honey bee health, with issues like climate change-driven drought and agricultural monocultures rapidly increasing. A better understanding of important nutrients to supplement during times of nutrient stress in the field could drastically improve honey bee health and reduce the increasing hive loss beekeepers are

experiencing. This research could also inform native pollinator conservation efforts about the role of the gut microbiome in bee health, which may prove useful to maintain healthy ecosystems.

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TABLES AND FIGURES

Figure 1.1 – Photo of a treatment hive with one brood frame pulled out. Yellow circle designates feeder frame. Telescoping cover and top cover (pictured on the left) are added to the top of the hive body, preventing exposure and robbing of resources such as supplemental feed or honey by bees from other hives or other organisms.

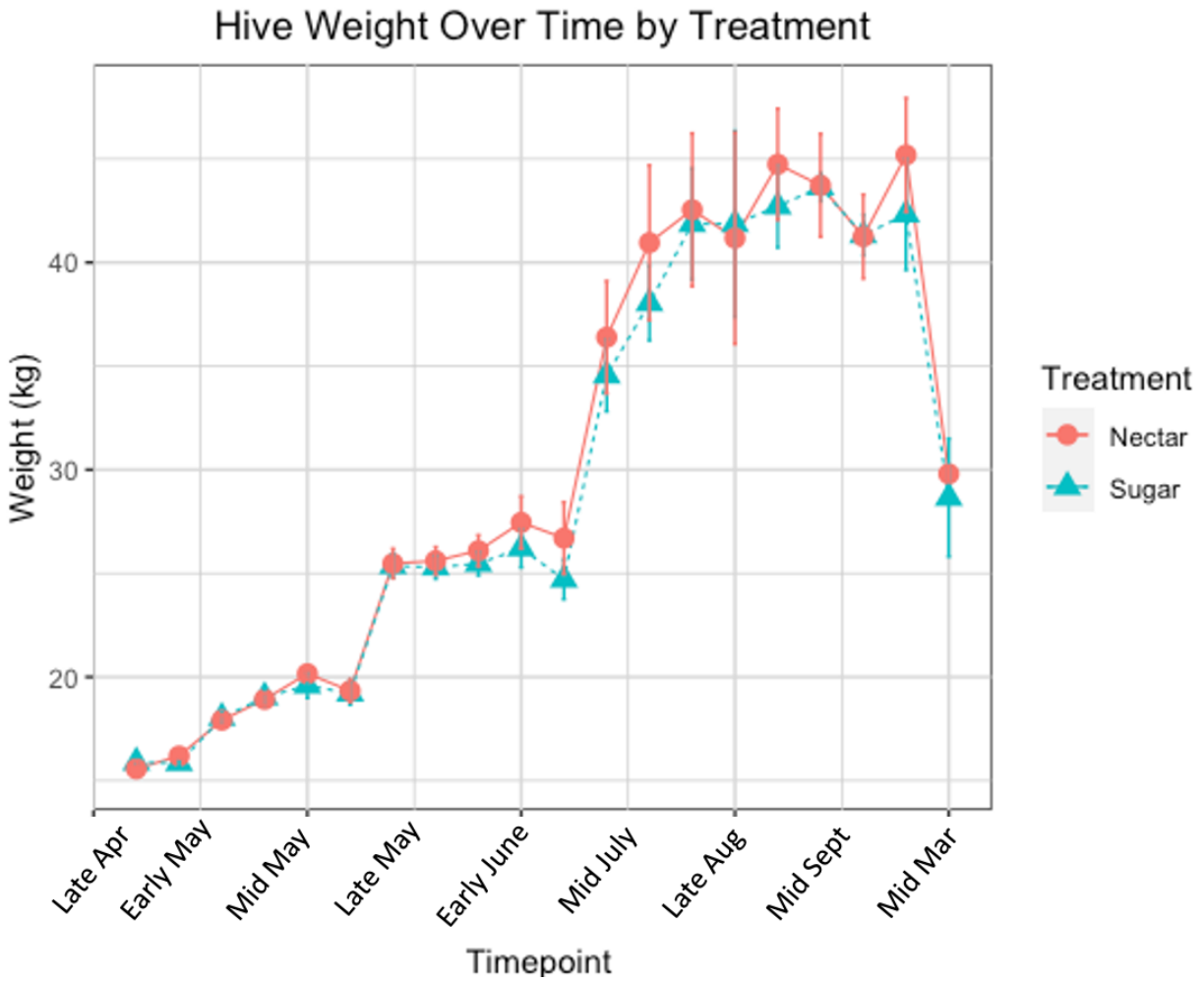


Figure 1.2 – Linear plot depicting hive weight over time by treatment, where the solid line is the "Sugar" treatment and the dashed line is the "Nectar" treatment. Hive weights did not differ over between treatments across all time points (linear mixed effect model, t-value = 0.62, p-value = 0.54).

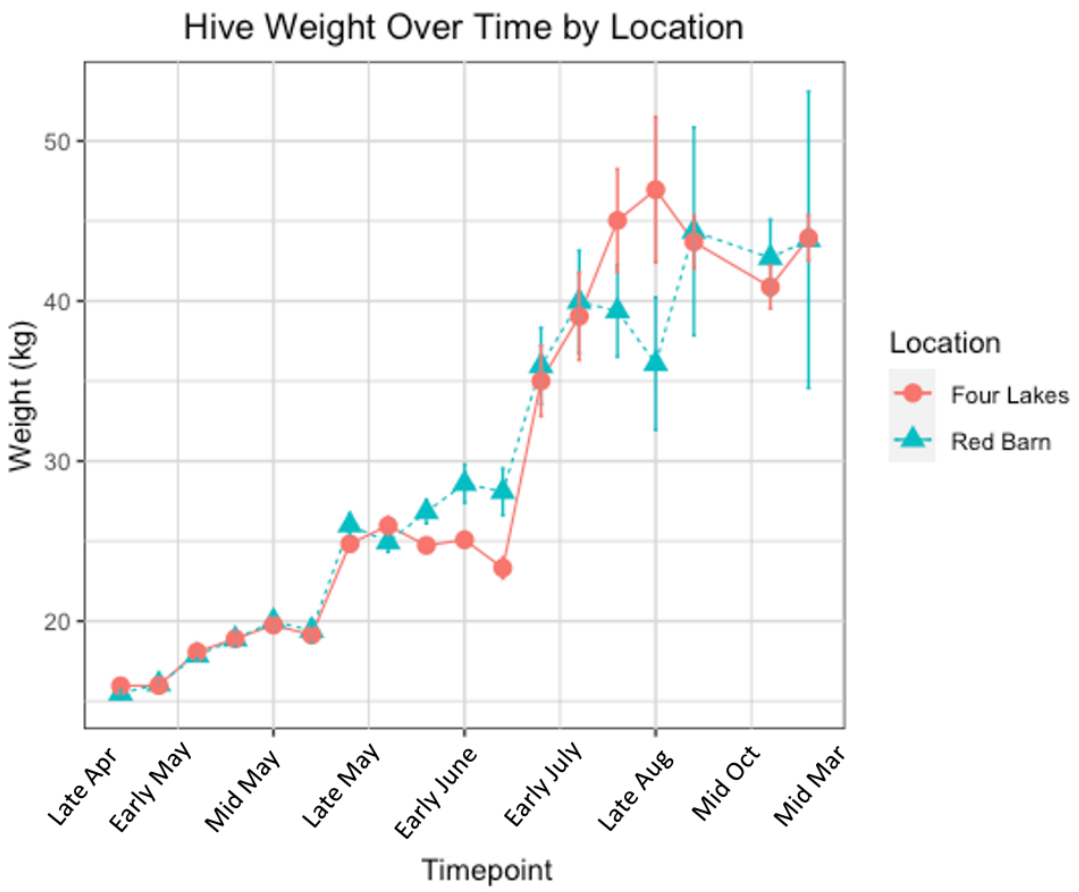


Figure 1.3 – Linear plot depicting hive weight over time by location, where the solid line are hives located at “Red Barn” and the dashed line are hives located at “Four Lakes”. Hive weights did not differ between location across all time points (linear mixed effect model, t-value = -1.45, p-value = 0.17). At three timepoints (June 1st, June 6th, and June 12th), Red Barn hives were significantly heavier than those at Four Lakes (see Figure 1.5, Table 1.4).

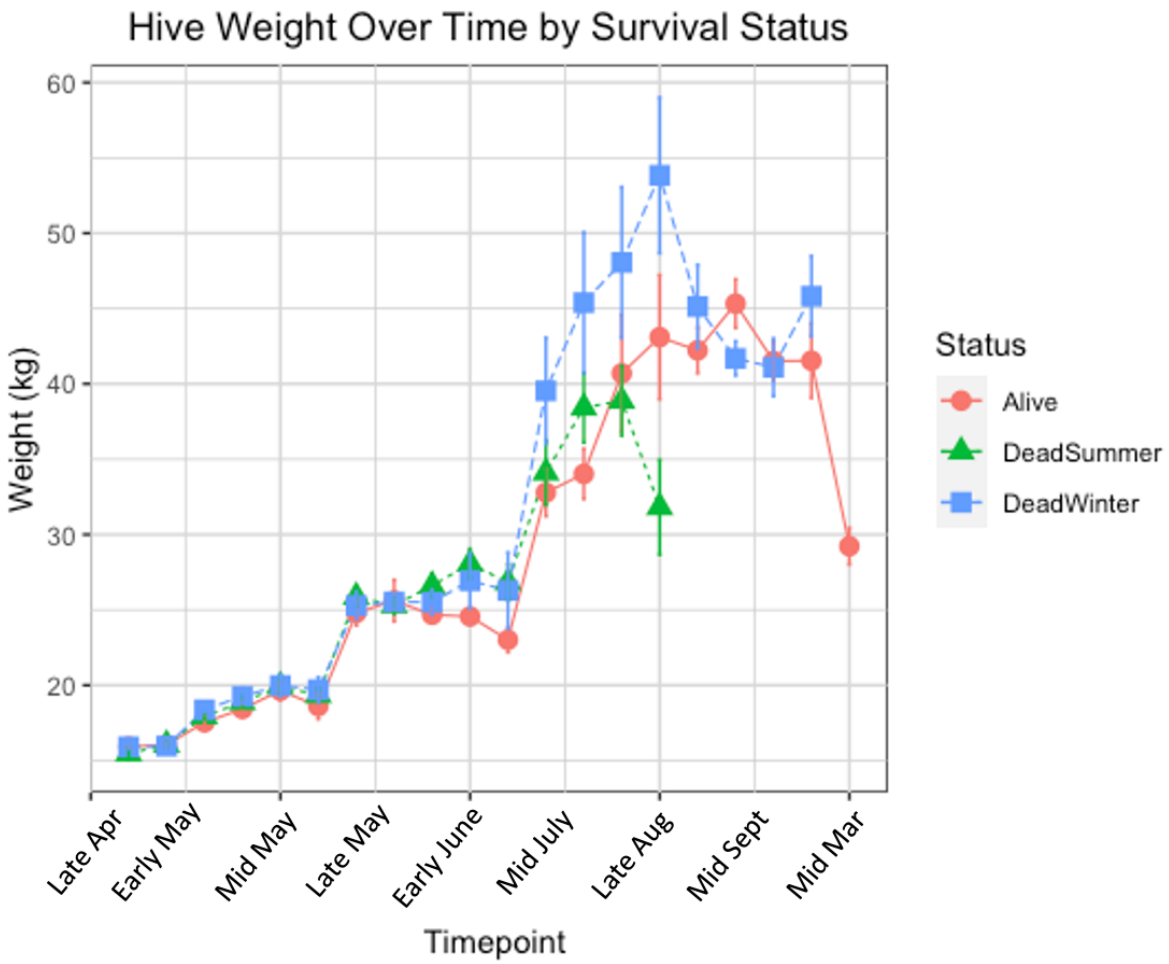


Figure 1.4 – Linear plot depicting hive weight over time by survival status, where the solid line are hives lost over winter, the dashed line are hives lost over summer, and the dotted line are hives that survived the year. Hive weights did not differ among survival status (linear mixed effect model, hives lost in summer t-value = -1.85, p-value = 0.09, hives lost in winter t-value = 1.62, p-value = 0.13). Survival status was significant on August 23rd (Figure 1.6; Kruskal-Wallis, chi-squared = 7.67, p-value = 0.02), where hives lost in the winter are heavier than hives lost in the summer (Wilcoxon Pairwise Test: p-value = 0.053) but not those that survived (Wilcoxon Pairwise Test: p-value = 0.19). Hives that survived were also not significantly different than those lost over summer (Wilcoxon Pairwise Test: p-value = 0.12).

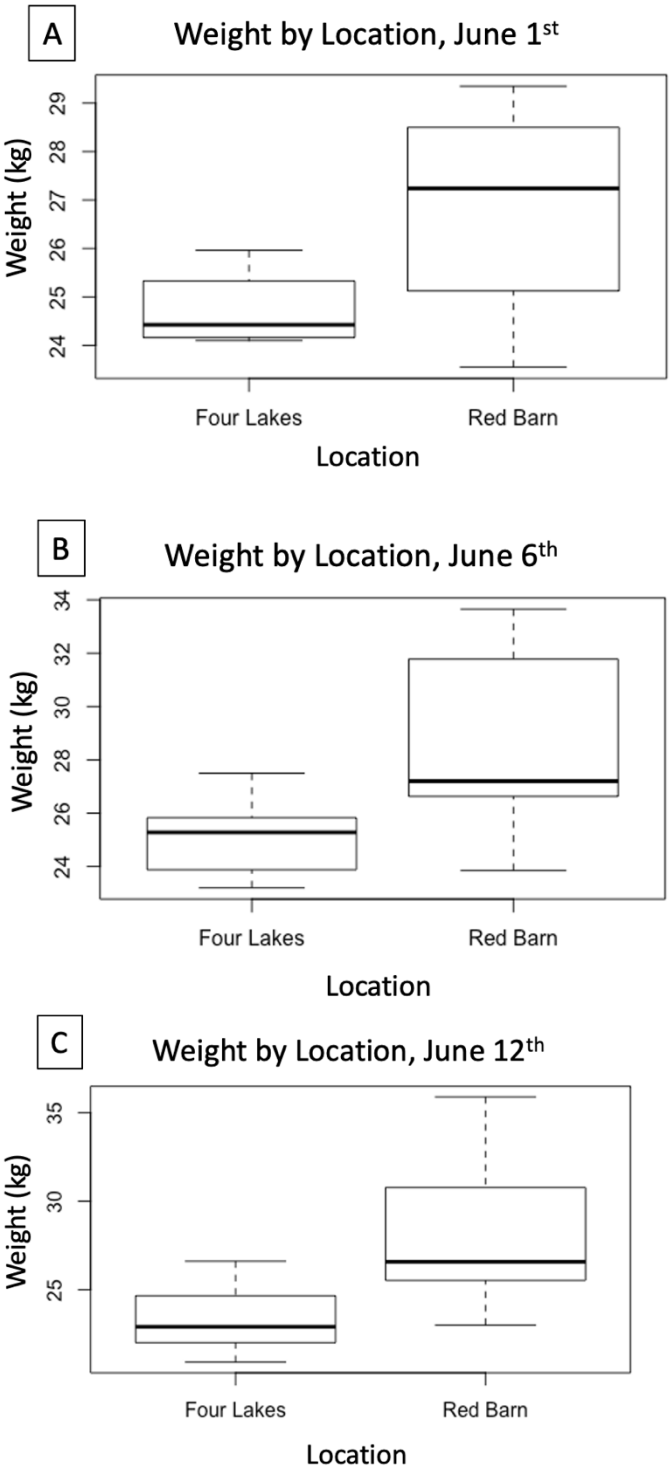


Figure 1.5. Box plots of average hive weight by location on June 1st (A), June 6th (B), and June 12th (C). Hive weight was significantly different by location on June 1st (Kruskal-Wallis, Chi-

Squared = 3.98, p-value = 0.046), June 6th (Kruskal-Wallis, Chi-Squared = 5.83, p-value = 0.016), and June 12th (Kruskal-Wallis, Chi-Squared = 6.89, p-value = 0.0087).

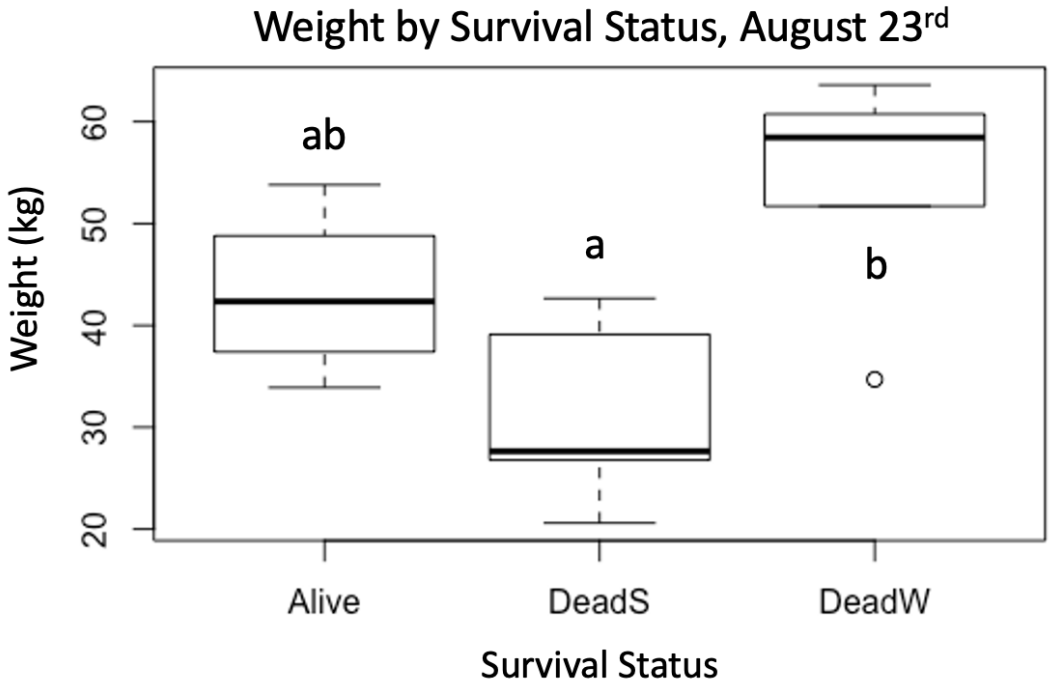


Figure 1.6. This boxplot shows hive weight by survival status on August 23rd where "Alive" are hives that survived the experiment, "DeadS" are hives lost over summer, and "DeadW" are hives lost over winter. Weights across survival status were significant overall at this timepoint (Kruskal-Wallis, chi-squared = 7.67, p-value = 0.02), where hives lost over winter were significantly different than hives lost over summer (p-value 0.053) but those lost over summer and winter were not significantly different from hives that survived (p-value = 0.12 and 0.19, respectively). The letters here indicate significance, where groups with different letters are significantly different from each other.

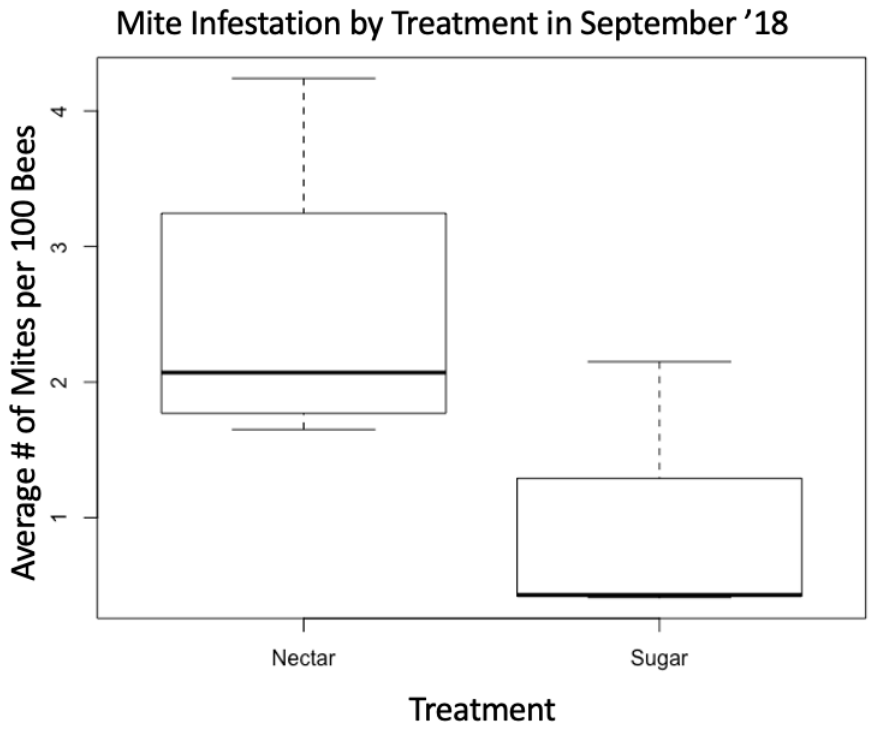


Figure 1.7. Box plot showing *Varroa destructor* mite infestation by two treatment groups “Nectar” and “Sugar” in September 2018. There was no significant difference between treatments (two-tailed t-test, t-value = 1.83, p-value = 0.13).

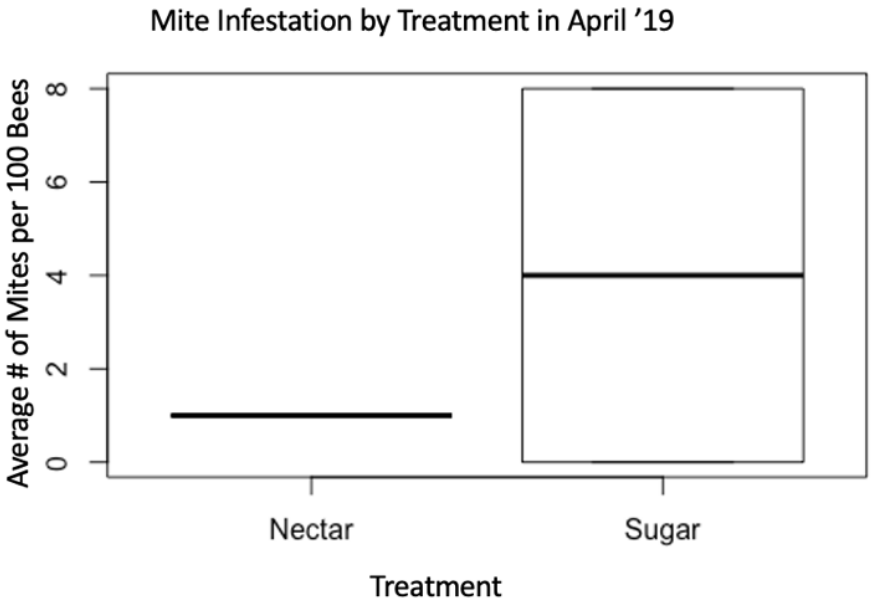


Figure 1.8. Box plot showing *Varroa destructor* mite infestation by two treatment groups “Nectar” and “Sugar” in April 2019. There was no significant difference between treatments (two-tailed t-test, t-value = -0.75, p-value = 0.59).

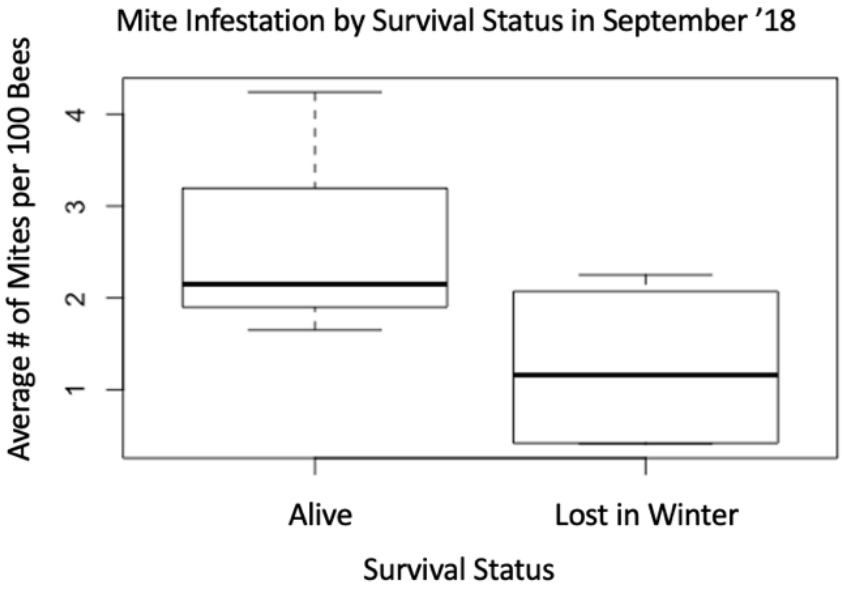


Figure 1.9. Box plot showing *Varroa destructor* mite infestation by two survival groups "Alive" and "Lost in Winter" in September 2018. There was no significant difference between survival groups (two-tailed t-test, t-value = 1.55, p-value = 0.2).

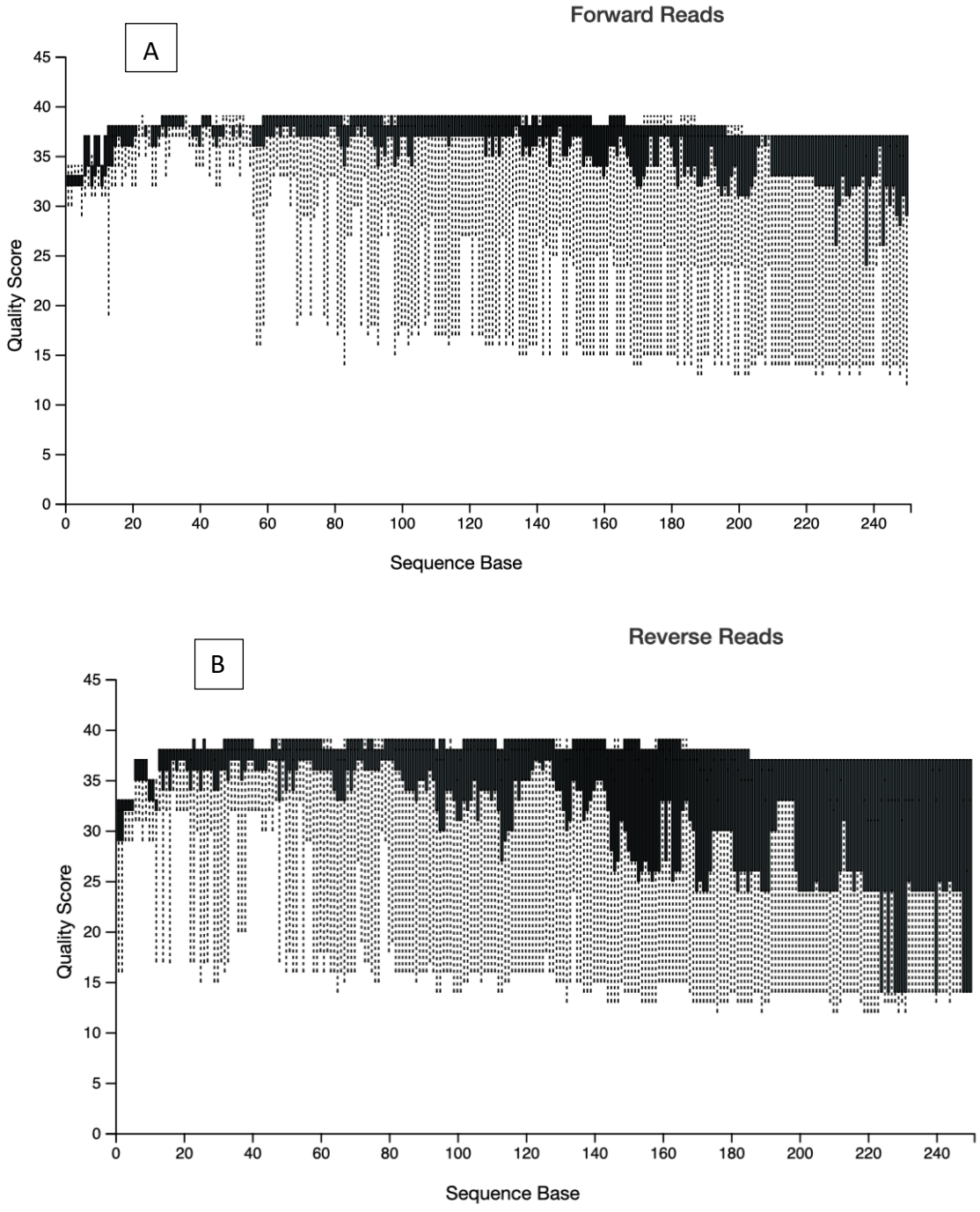


Figure 2.1 Quality scores of DNA amplicon sequences before trimming using DADA2 on the forward reads (A) and reverse (B) reads.

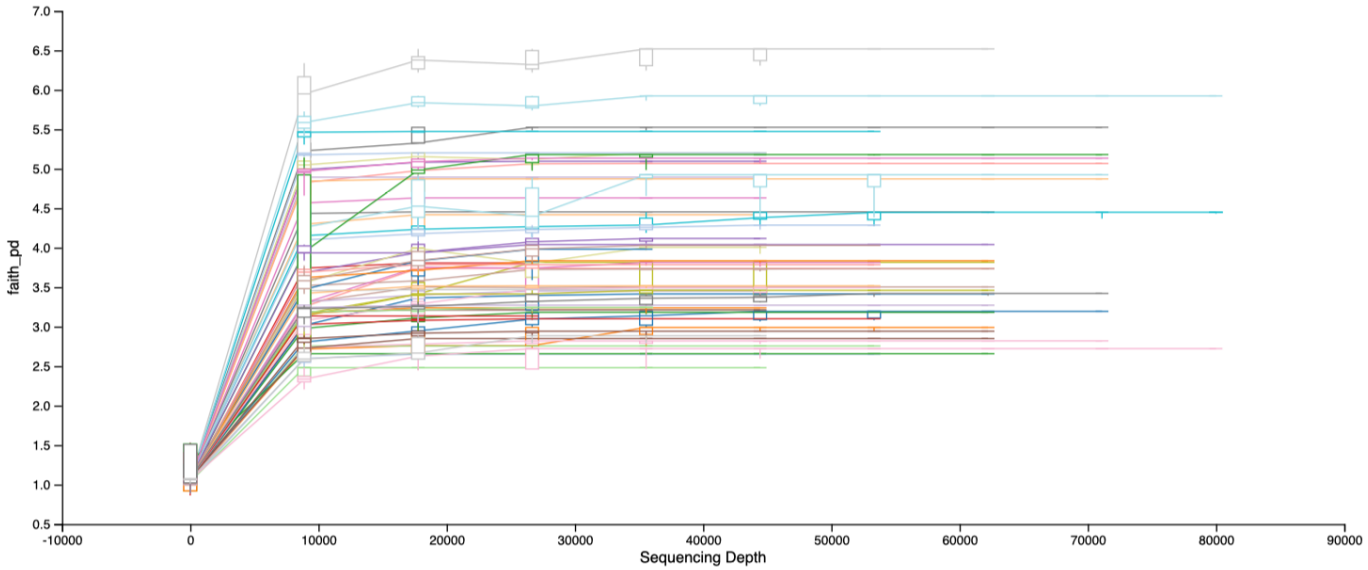


Figure 2.2. Alpha rarefaction plot. This graph shows how sequencing depth influences the amount of diversity captured when visualizing Faith's Phylogenetic Diversity. Each individual line is a sample. All samples were rarefied at maximum sequencing depth of 30834 sequences per sample.

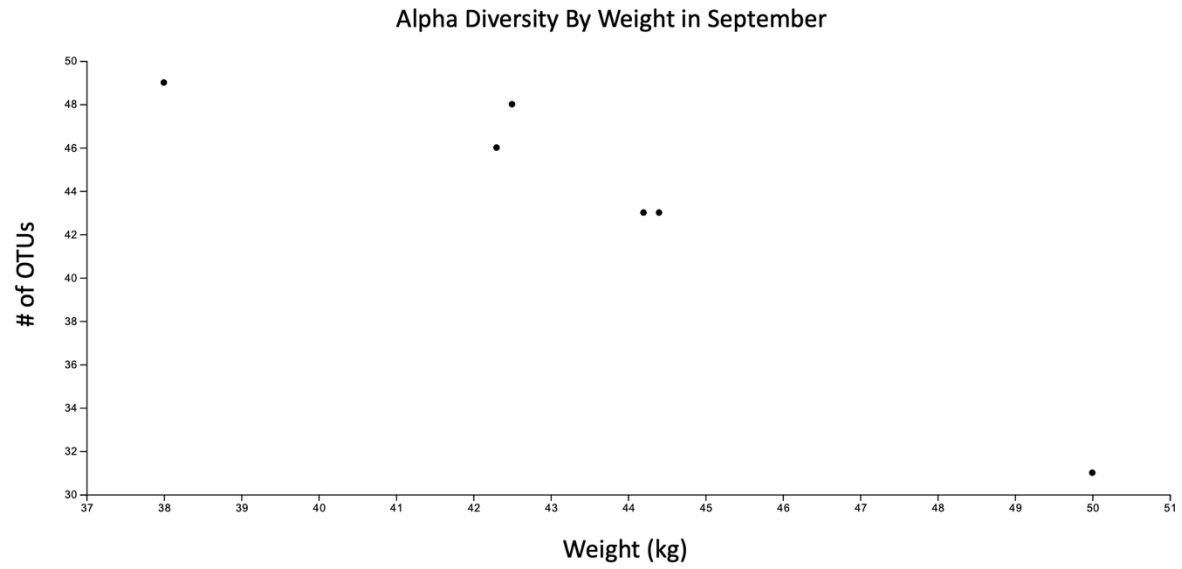


Figure 2.3. Scatter plot where each dot is a single hive. X-axis shows hive weight (kg) and Y-axis shows OTU richness (# of OTUs). There was a negative correlation here between hive weight and bacterial richness (observed OTUs, Spearman Rank Test, r_s = -0.93, p-value = 0.007).

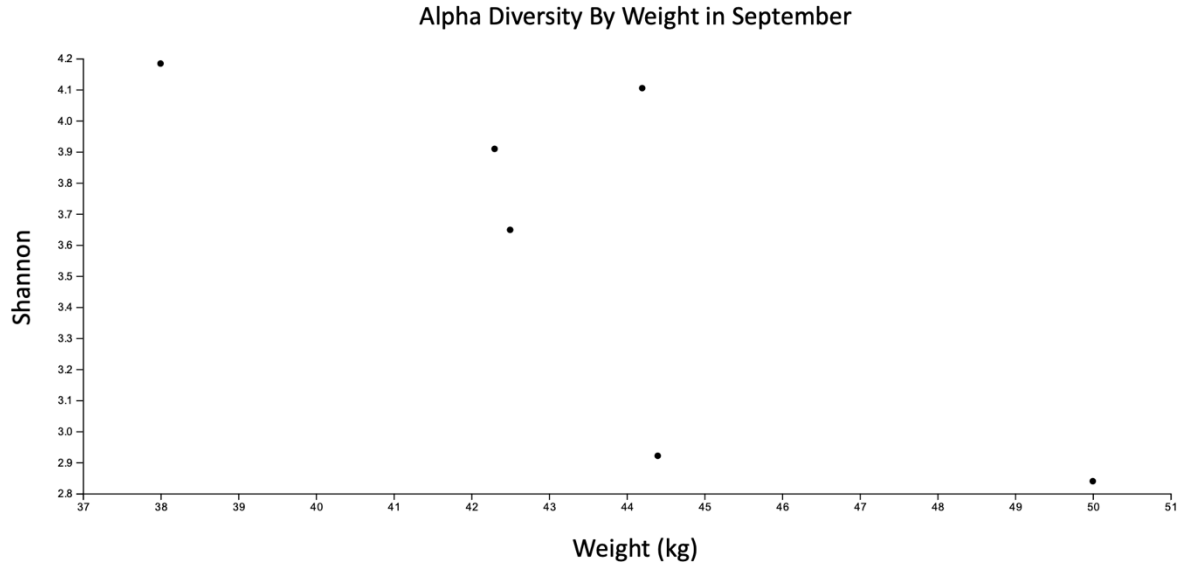


Figure 2.4. Scatter plot where each dot is a single hive. X-axis shows hive weight (kg), and Y-axis shows Shannon diversity metric. There was a negative correlation between hive weight and bacterial diversity (Shannon, Spearman Rank Test, r_s = -0.83, p-value = 0.004).

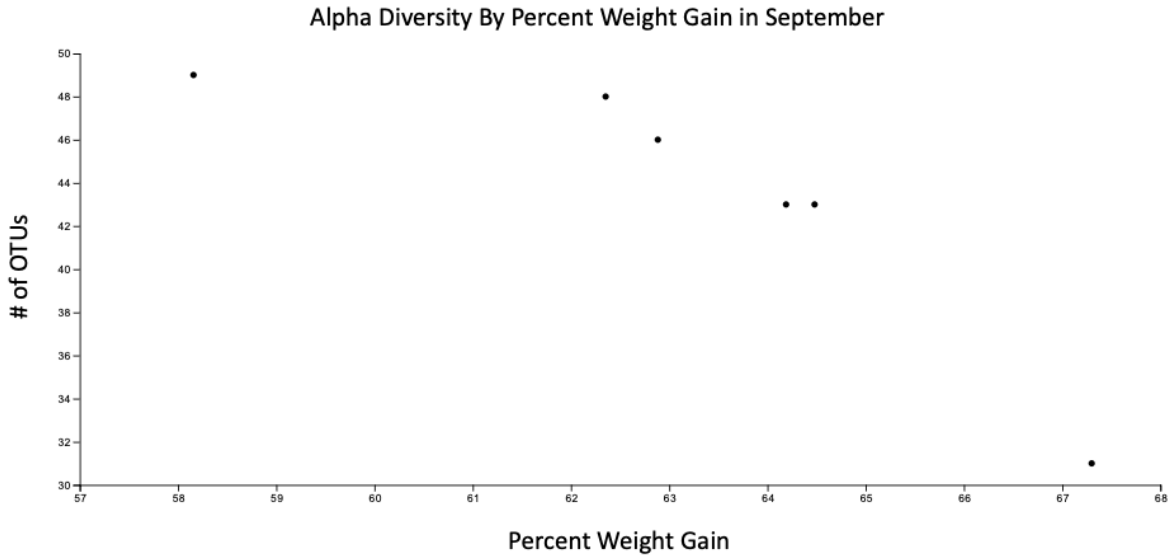


Figure 2.5. Scatter plot where each dot is a single hive. X-axis shows percent weight gained since experiment start in April, and Y-axis shows # of OTUs. Hives that gained less weight throughout the duration of the experiment had higher OTU richness than hives that gained more weight (observed OTUs, Spearman Rank Test, r_s = value -0.99, p-value = 0.0003).

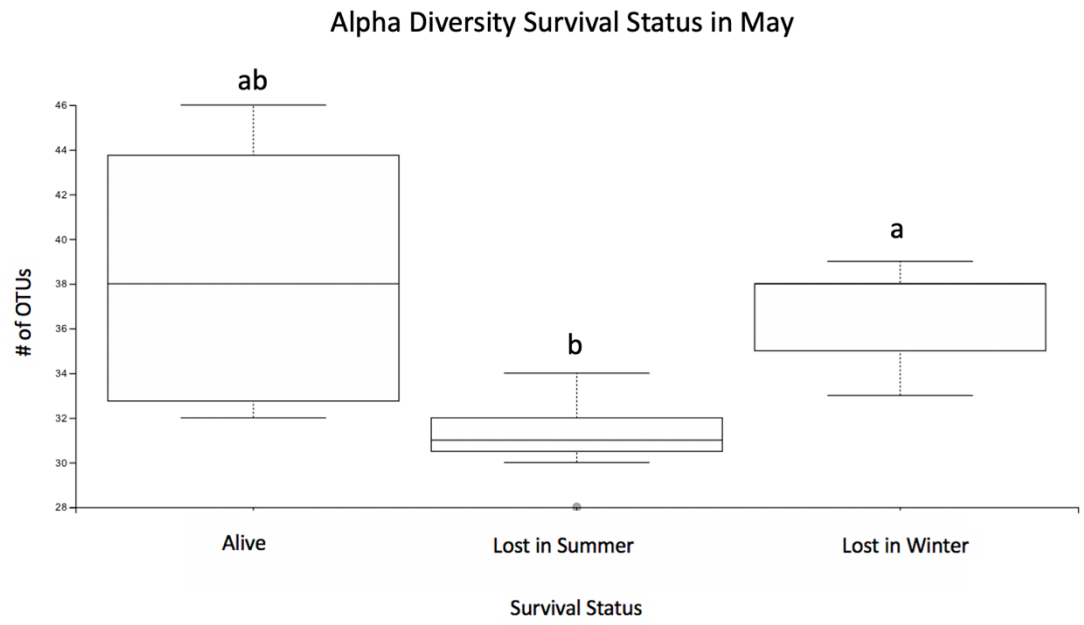


Figure 2.6. Box plot depicting OTU richness alpha diversity differences between survival status in May. Hives that died in the summer had significantly lower diversity than those that survived the experiment (observed OTUs, Kruskal-Wallis, H = 4.03 p-value = 0.045) and those that were lost later over winter (observed OTUs, Kruskal-Wallis, H = 6.90, p-value = 0.0086). Hives lost over winter and hives that survived were not significantly different (observed OTUS, Kruskal-Wallis, H = 0.009, p-value = 0.92).

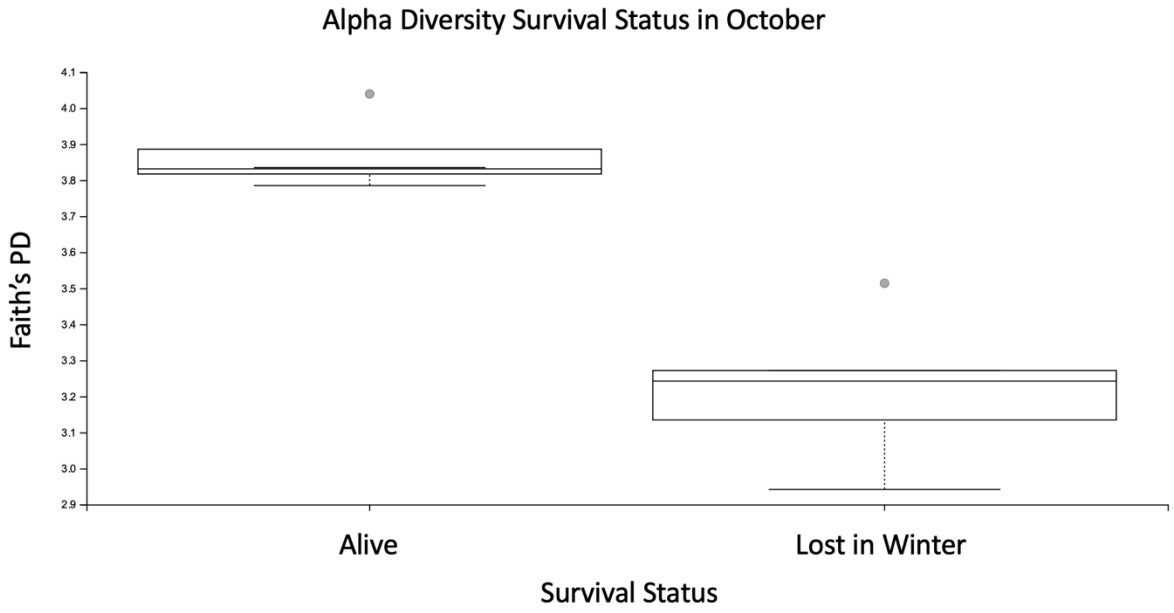


Figure 2.7. Box plot depicting OTU richness differences between survival status in October. Hives lost over winter had significantly lower diversity that hives that survived the winter (Faith's phylogenetic diversity, Kruskal-Wallis, H = 6, p-value = 0.014).

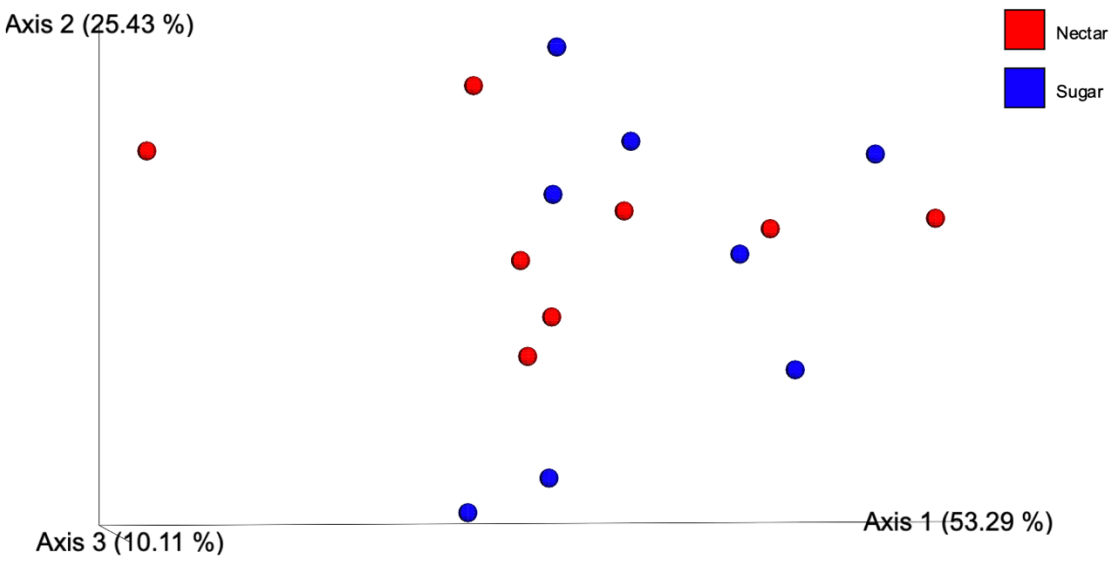


Figure 2.8. Principal Coordinate Analysis Plot using Weighted Unifrac dissimilarity matrix to visualize differences in microbiome community between treatment groups of hives at the May Timepoint. There were no significant differences between treatment and microbiome at any timepoint (all p-values > 0.05)

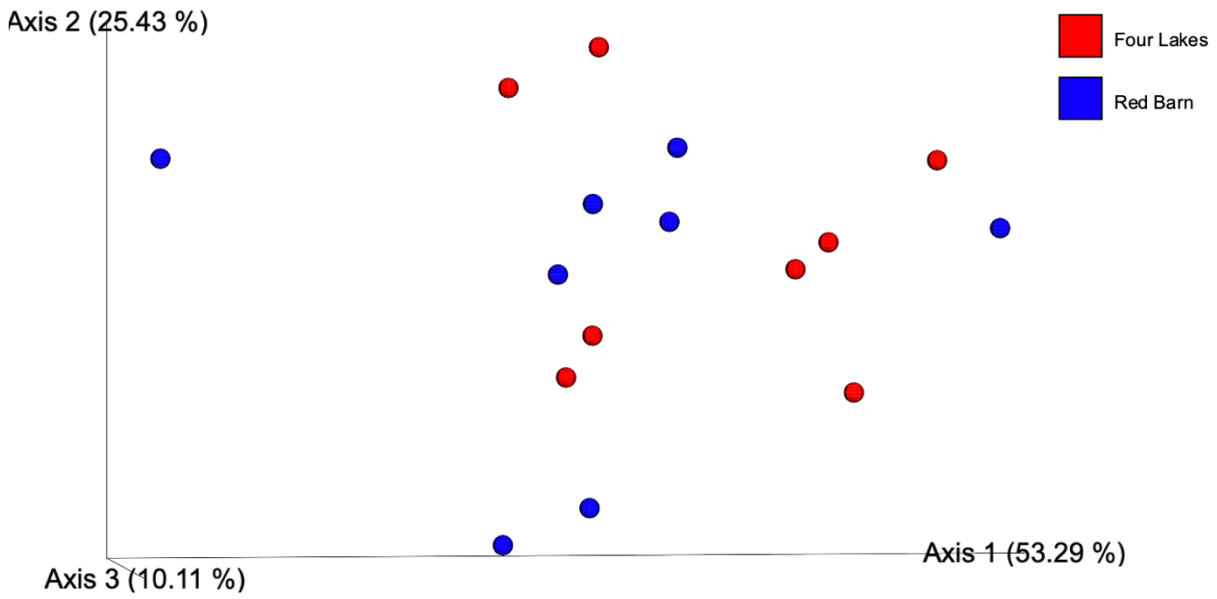


Figure 2.9. Principal Coordinate Analysis Plot using Weighted Unifrac dissimilarity matrix to visualize differences in microbiome community between location of hives at the May Timepoint. There were no significant differences between location and microbiome at any timepoint (all p-values > 0.05)

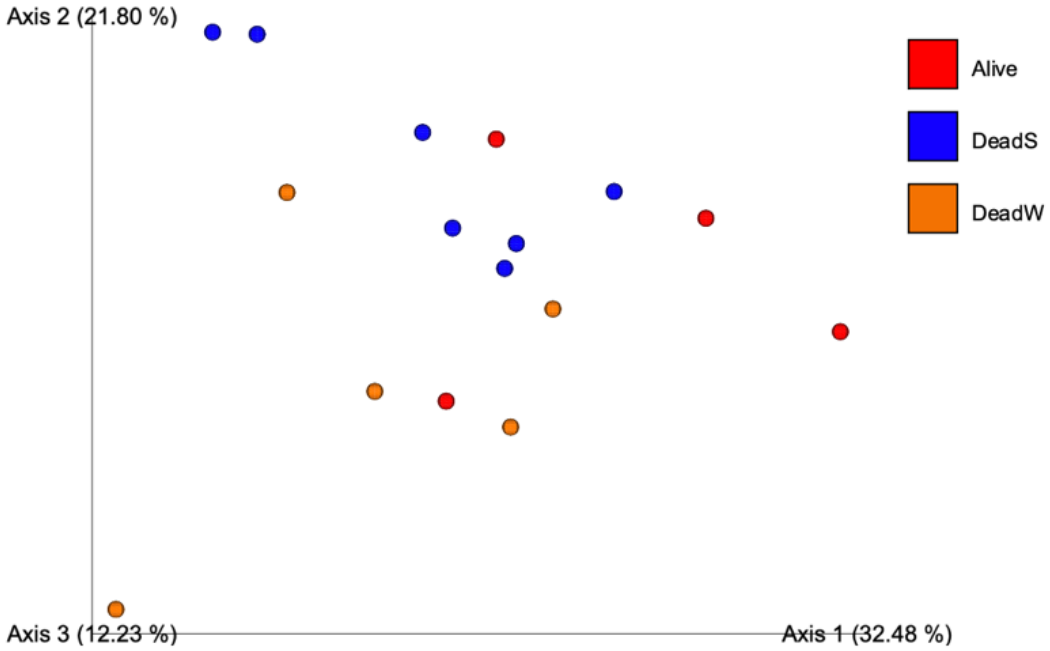


Figure 2.10. Principal Coordinate Analysis Plot using Bray-Curtis dissimilarity matrix to visualize differences in microbiome community between survival status of hives at the April Timepoint. All three survival statuses had significantly different microbiome structures (Bray-Curtis, PERMANOVA, pseudo-F = 1.98, p- value = 0.025).

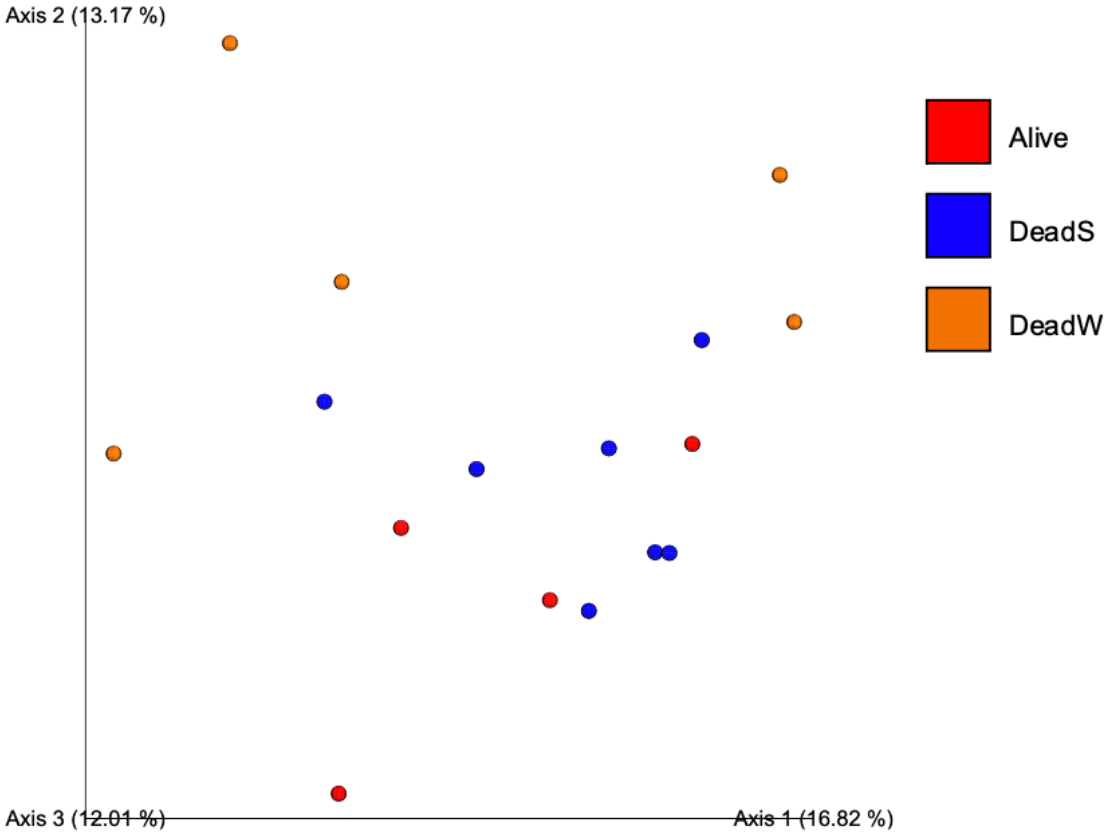


Figure 2.11. Principal Coordinate Analysis Plot using Jaccard distance matrix to visualize differences in microbiome community between survival status of hives at the May timepoint. Among three hive survival statuses, there was difference in microbiome composition (Jaccard, PERMANOVA, pseudo-F = 1.34, p-value = 0.047).

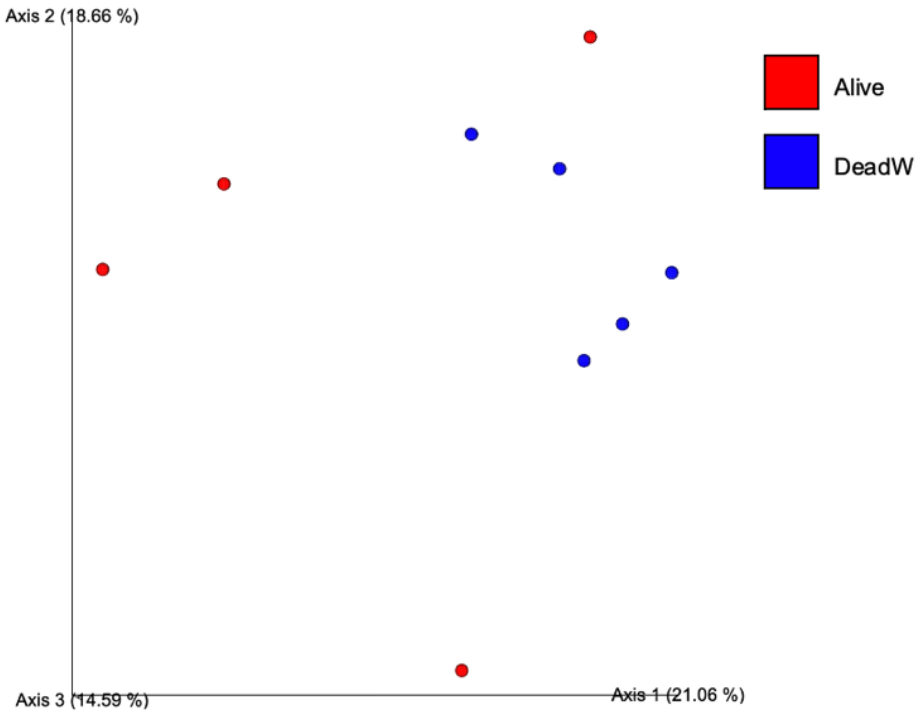


Figure 2.12. Principal Coordinate Analysis Plot using Jaccard distance matrix to visualize differences in microbiome community between survival status of hives at the August timepoint. Hives that survived and those that were lost over winter had statistically different microbiome compositions (Jaccard, PERMANOA, pseudo-F = 1.4, p-value = 0.044).

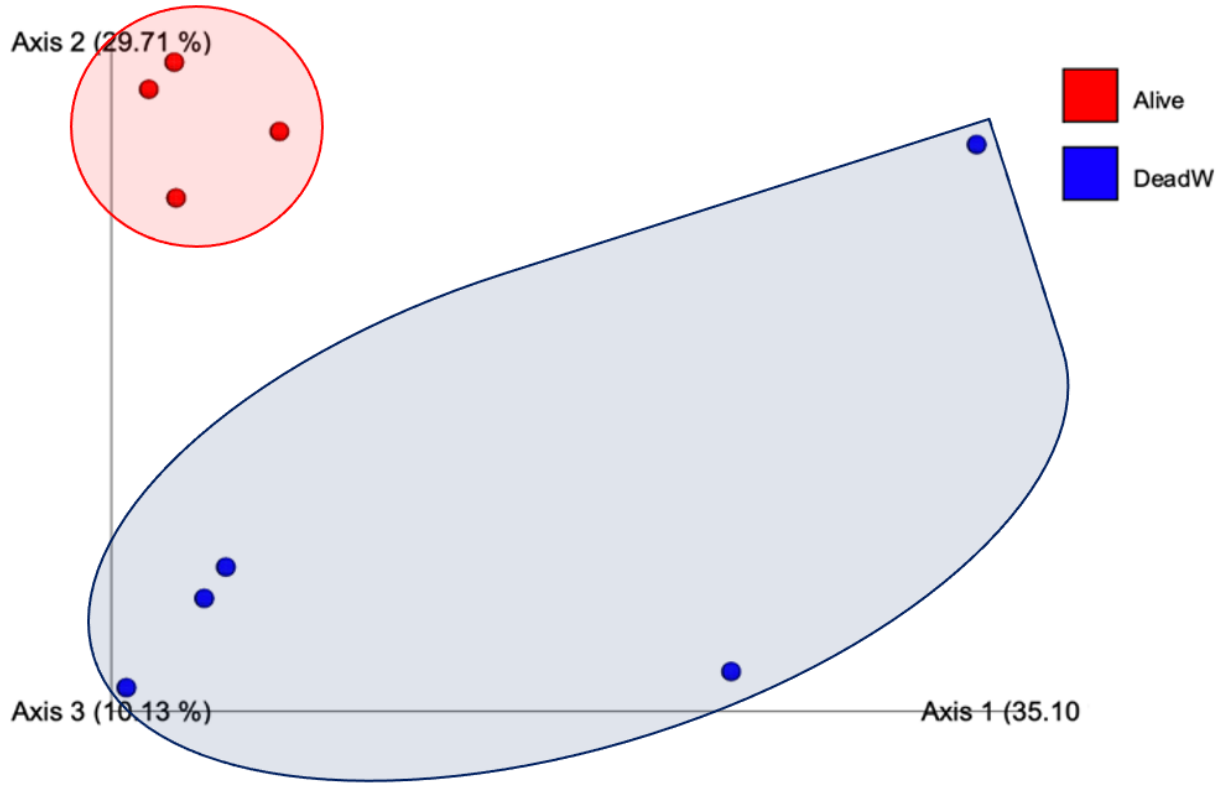
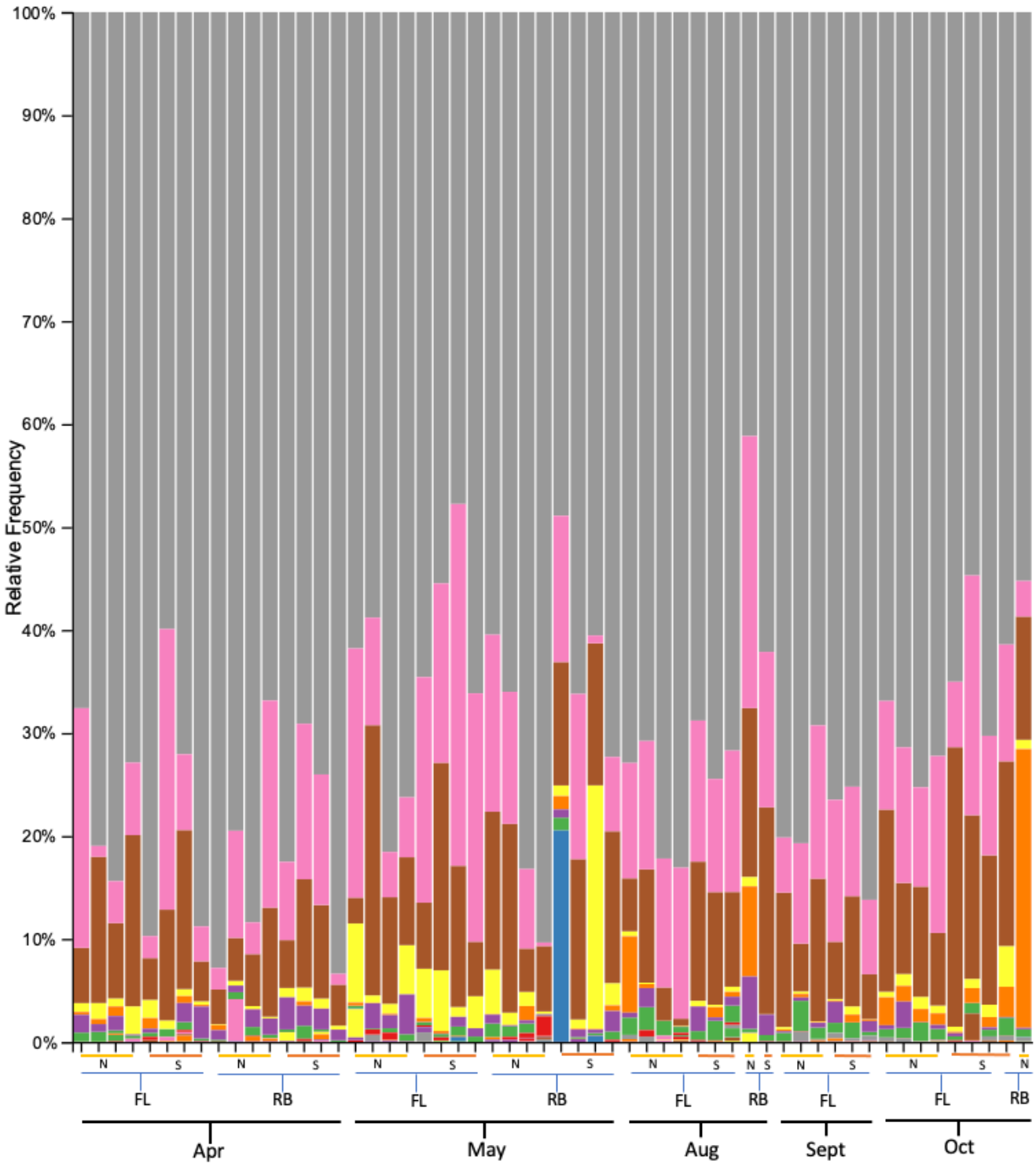
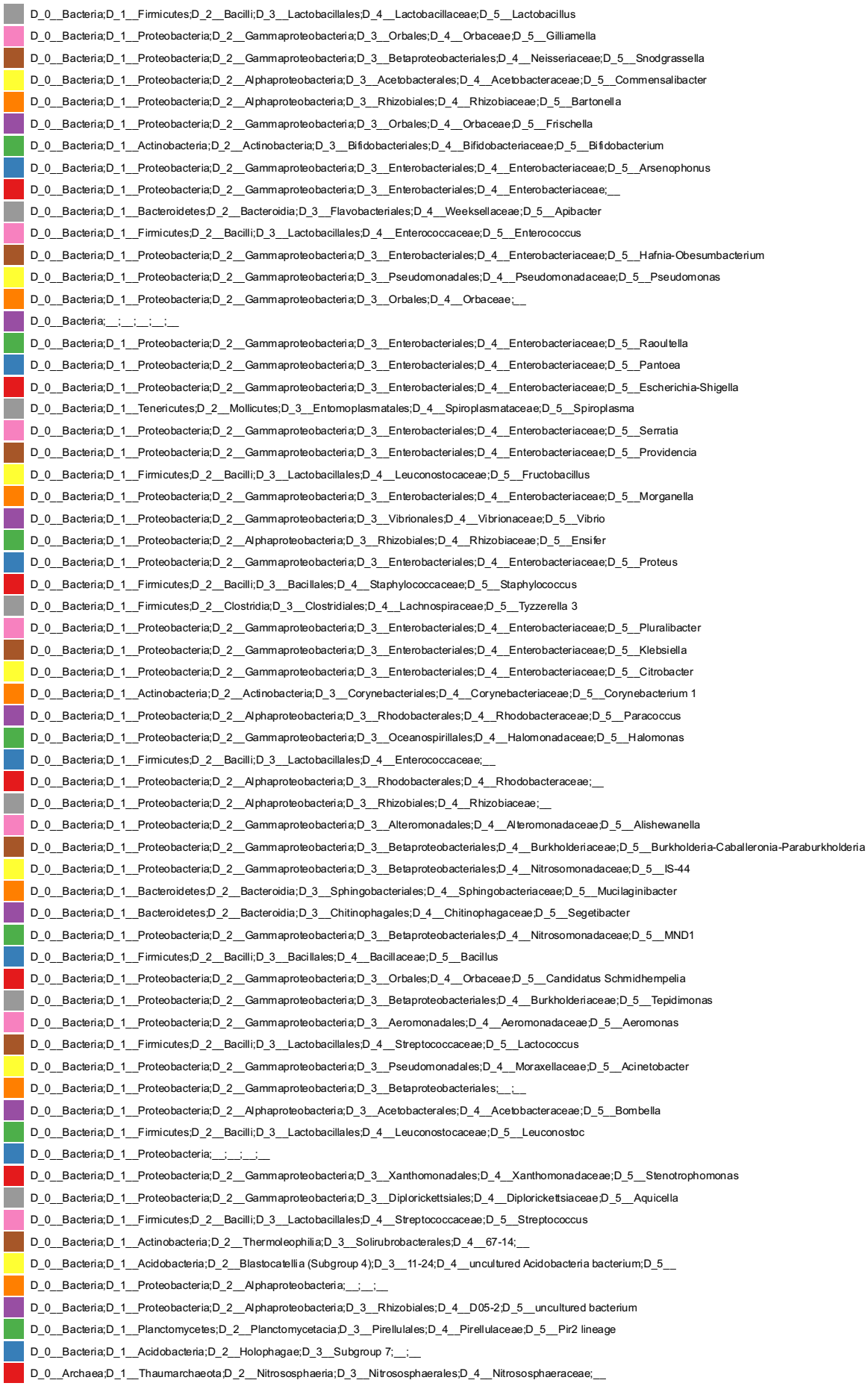


Figure 2.13. Principal Coordinate Analysis Plot using Unweighted Unifrac distance matrix to visualize differences in microbiome community between survival status of hives at the October timepoint. Hives lost over winter had significantly different microbiome composition than hives that survived at the October timepoint (Unweighted Unifrac, PERMANOVA, pseudo-F = 2.54, p-value = 0.023).


100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
Relative Frequency
N
S
FL
RB
Apr
May
Aug
Sept
Oct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D_0__Bacteria;D_1__Proteobacteria;D_2__Gammaproteobacteria;D_3__Orbales;D_4__Orbaceae;D_5__Gilliamella
D_0__Bacteria;D_1__Proteobacteria;D_2__Gammaproteobacteria;D_3__Betaproteobacteriales;D_4__Neisseriaceae;D_5__Snodgrassella
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D_0__Bacteria;D_1__Proteobacteria;D_2__Alphaproteobacteria;D_3__Rhizobiales;D_4__Rhizobiaceae;D_5__Bartonella
D_0__Bacteria;D_1__Proteobacteria;D_2__Gammaproteobacteria;D_3__Orbales;D_4__Orbaceae;D_5__Frischella
D_0__Bacteria;D_1__Actinobacteria;D_2__Actinobacteria;D_3__Bifidobacteriales;D_4__Bifidobacteriaceae;D_5__Bifidobacterium
D_0__Bacteria;D_1__Proteobacteria;D_2__Gammaproteobacteria;D_3__Enterobacteriales;D_4__Enterobacteriaceae;D_5__Arsenophonus
D_0__Bacteria;D_1__Proteobacteria;D_2__Gammaproteobacteria;D_3__Enterobacteriales;D_4__Enterobacteriaceae;__
D_0__Bacteria;D_1__Bacteroidetes;D_2__Bacteroidia;D_3__Flavobacteriales;D_4__Weeksellaceae;D_5__Apibacter
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D_0__Bacteria;D_1__Proteobacteria;D_2__Gammaproteobacteria;D_3__Orbales;D_4__Orbaceae;__
D_0__Bacteria;__;__;__;__;__
D_0__Bacteria;D_1__Proteobacteria;D_2__Gammaproteobacteria;D_3__Enterobacteriales;D_4__Enterobacteriaceae;D_5__Raoultella
D_0__Bacteria;D_1__Proteobacteria;D_2__Gammaproteobacteria;D_3__Enterobacteriales;D_4__Enterobacteriaceae;D_5__Pantoea
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D_0__Bacteria;D_1__Tenericutes;D_2__Mollicutes;D_3__Entomoplasmatales;D_4__Spiroplasmataceae;D_5__Spiroplasma
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D_0__Bacteria;D_1__Proteobacteria;D_2__Gammaproteobacteria;D_3__Enterobacteriales;D_4__Enterobacteriaceae;D_5__Citrobacter
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D_0__Bacteria;D_1__Proteobacteria;D_2__Alphaproteobacteria;D_3__Rhodobacterales;D_4__Rhodobacteraceae;D_5__Paracoccus
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D_0__Bacteria;D_1__Actinobacteria;D_2__Thermoleophilia;D_3__Solirubrobacterales;D_4__67-14;__
D_0__Bacteria;D_1__Acidobacteria;D_2__Blastocatellia (Subgroup 4);D_3__11-24;D_4__uncultured Acidobacteria bacterium;D_5__
D_0__Bacteria;D_1__Proteobacteria;D_2__Alphaproteobacteria;__;__;__
D_0__Bacteria;D_1__Proteobacteria;D_2__Alphaproteobacteria;D_3__Rhizobiales;D_4__D05-2;D_5__uncultured bacterium
D_0__Bacteria;D_1__Planctomycetes;D_2__Planctomycetacia;D_3__Pirellulales;D_4__Pirellulaceae;D_5__Pir2 lineage
D_0__Bacteria;D_1__Acidobacteria;D_2__Holophagae;D_3__Subgroup 7;__;__
D_0__Archaea;D_1__Thaumarchaeota;D_2__Nitrososphaeria;D_3__Nitrososphaerales;D_4__Nitrososphaeraceae;__

Figure 2.14. Bar plot showing relative abundance of bacterial groups at the Genus level. This bar plot shows nectar and sugar treated hives over all time, where “FL” represents hives located at Four Lakes, “RB” represents hives at Red Barn, “N” represents hives fed supplemental nectar and pollen nutrient mixture, and “S” represents hives fed the control sugar-water mixture.

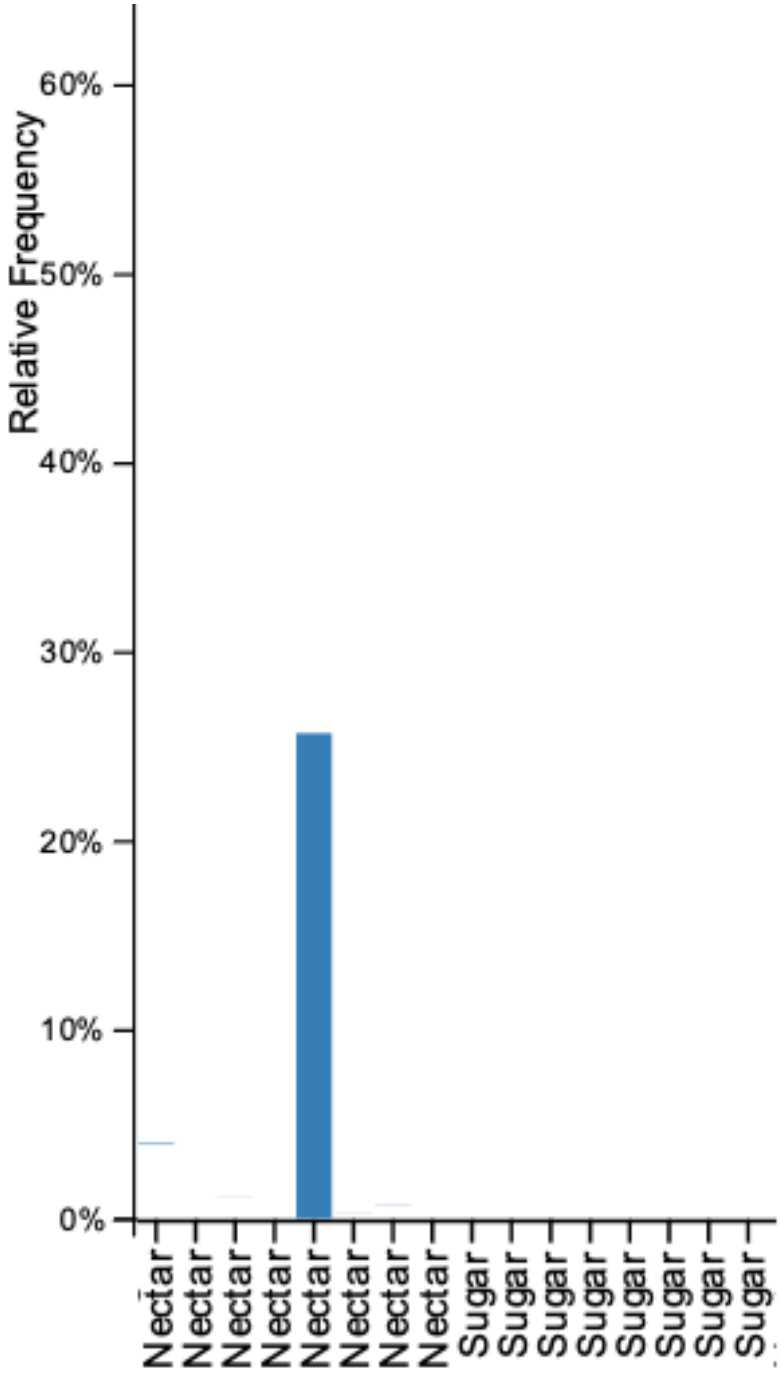

Figure 2.15. Relative abundance bar plot showing relative abundance of *Arsenophonus* across treatment groups at the May timepoint. *Arsenophonus* was significantly higher in Nectar treated hives than Sugar treated hives (Nectar Mean = 2.6%, Sugar Mean = 0%, LDA = 4.45, p-value = 0.011).

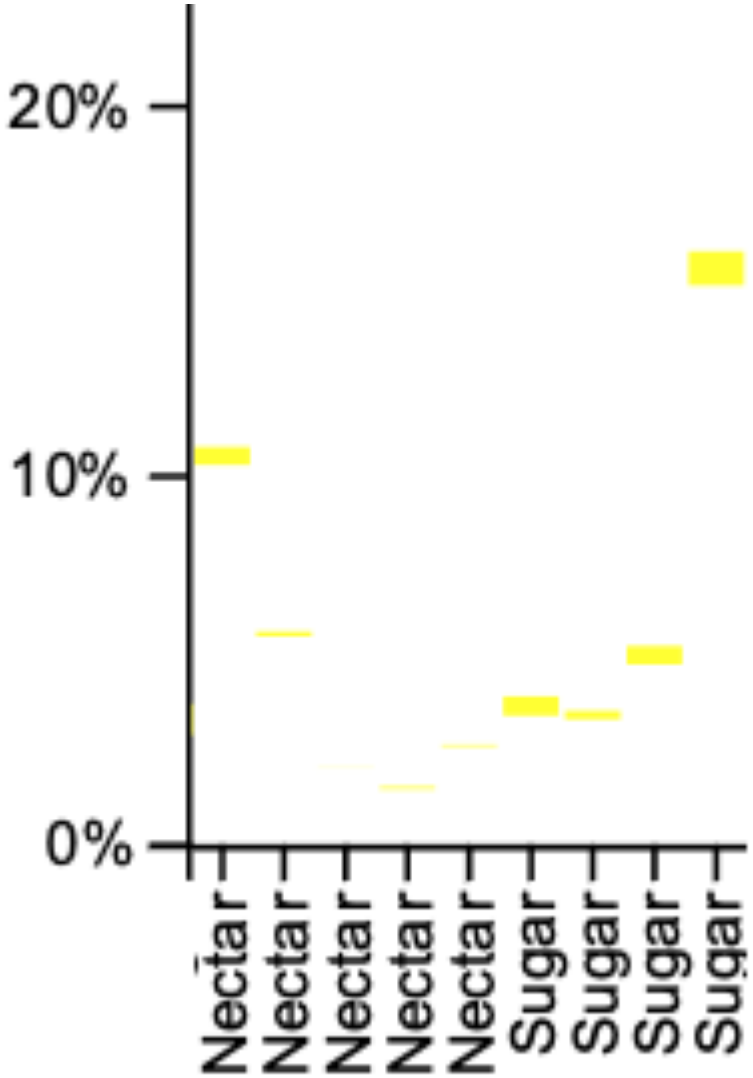

Figure 2.16. Relative abundance bar plot showing relative abundance of *Commensalibacter* at the August timepoint across treatment groups. *Commensalibacter* was significantly higher in

Sugar hives, although it was in low relative abundance overall in both treatments (Nectar Mean = 0.15%, Sugar Mean = 0.54%, LDA = 3.66, p-value 0.027).

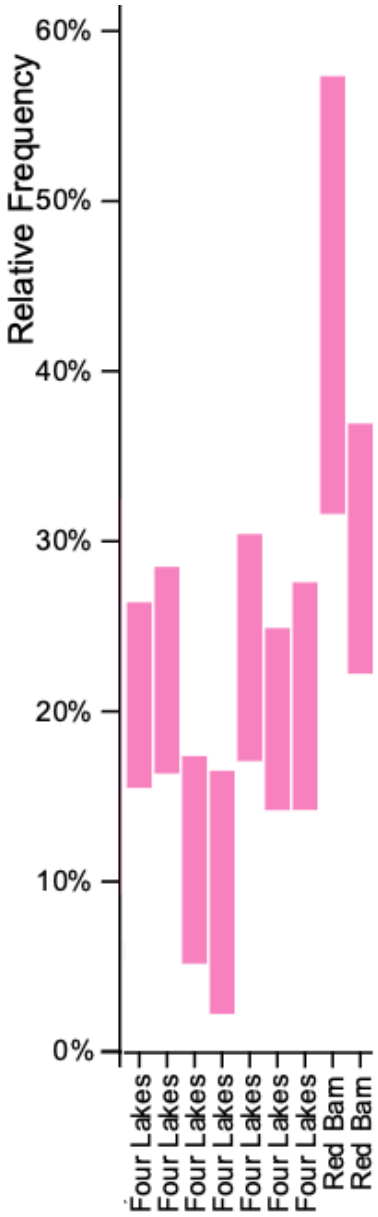

Figure 2.17. Relative abundance bar plot showing relative abundance of *Gilliamella* at the August timepoint across apiary locations. *Gilliamella* had a higher relative abundance at Red Barn location than Four lakes (Four Lakes mean = 12.8%, Red Barn mean = 20.7%, LDA = 4.66, p-value = 0.04).

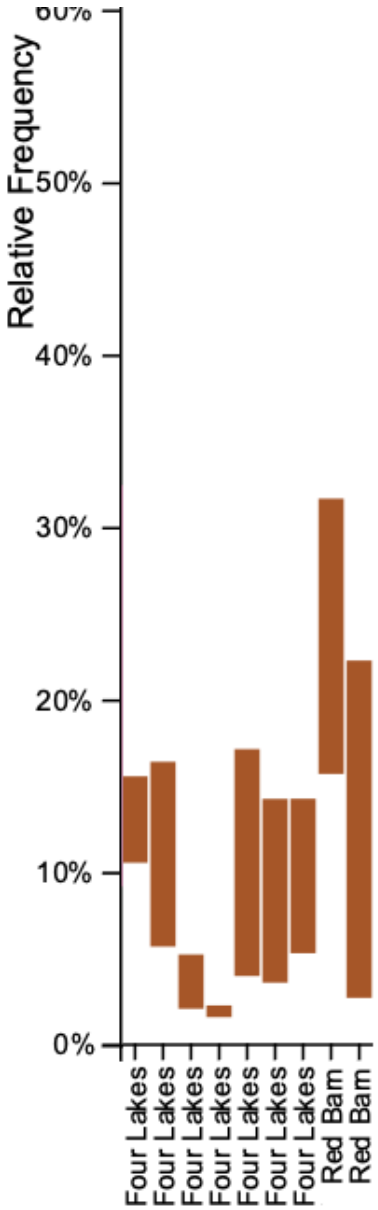

Figure 2.18. Relative abundance bar plot showing relative abundance of *Snodgrasella* at the August timepoint across apiary locations. *Snodgrasella* had a higher relative abundance at Red Barn location than Four lakes (Four Lakes mean = 7.7%, Red Barn mean = 18.2%, LDA = 4.78, p-value = 0.04).

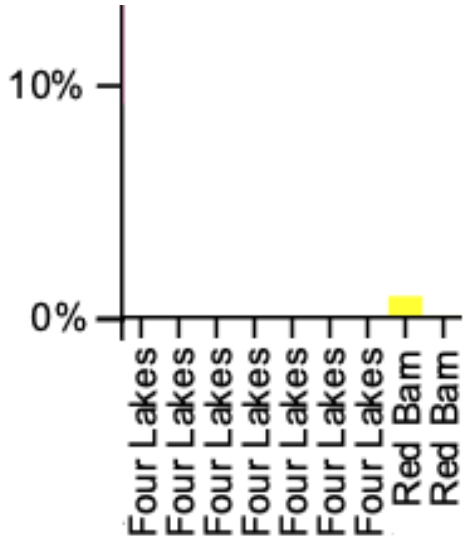

Figure 2.19. Relative abundance bar plot showing relative abundance of *Pseudomonas* at the August timepoint across apiary locations. *Pseudomonas* had a higher relative abundance at Red Barn location than Four lakes (Four Lakes mean = 0%, Red Barn mean = 0.43%, LDA = 3.97, p-value = 0.005).

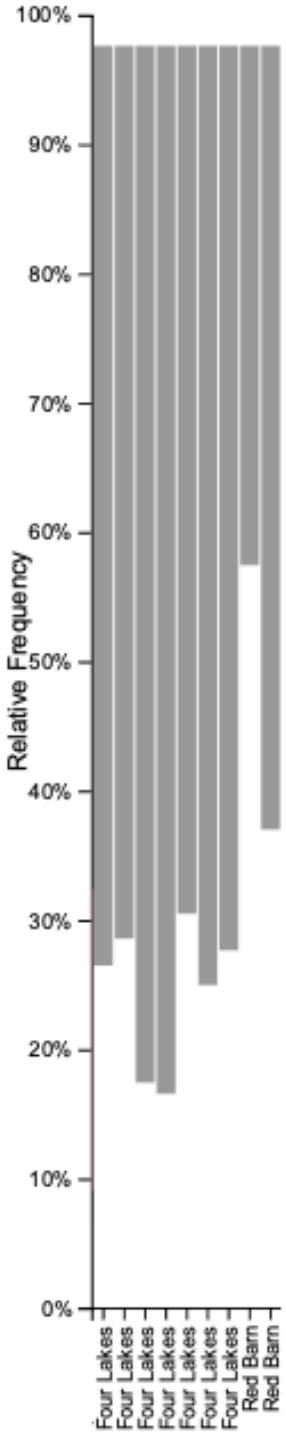

Figure 2.20. Relative abundance bar plot showing relative abundance of *Lactobacillus* at the August timepoint across apiary locations*. Lactobacillus* was higher at Four Lakes than Red Barn at this timepoint (Four Lakes mean = 75%, Red Barn mean = 52%, LDA = 5.05, p-value = 0.04).

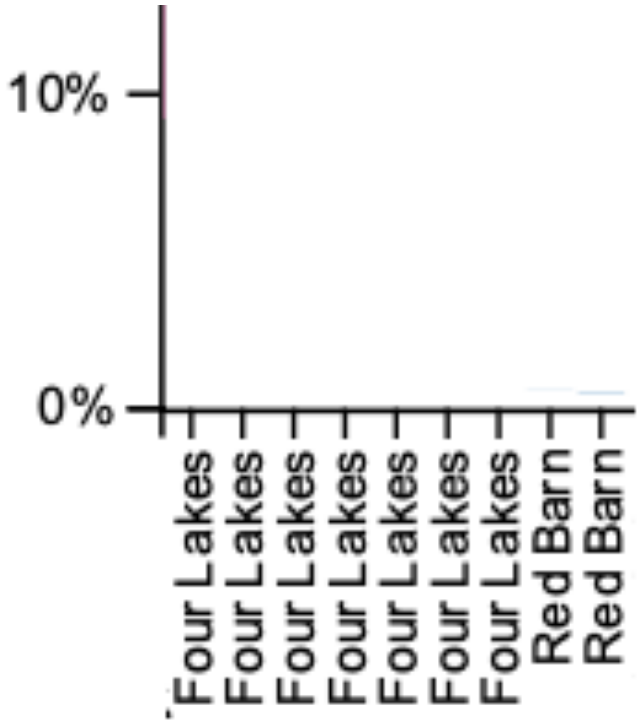

Figure 2.21. Relative abundance bar plot showing relative abundance of *Arsenophonus* in October across apiary locations. *Arsenophonus* was higher in Red Barn hives than Four Lake hives at this timepoint (Four Lakes mean = 0%, Red Barn mean = 0.017%, LDA = 4.85, p-value = 0.005).

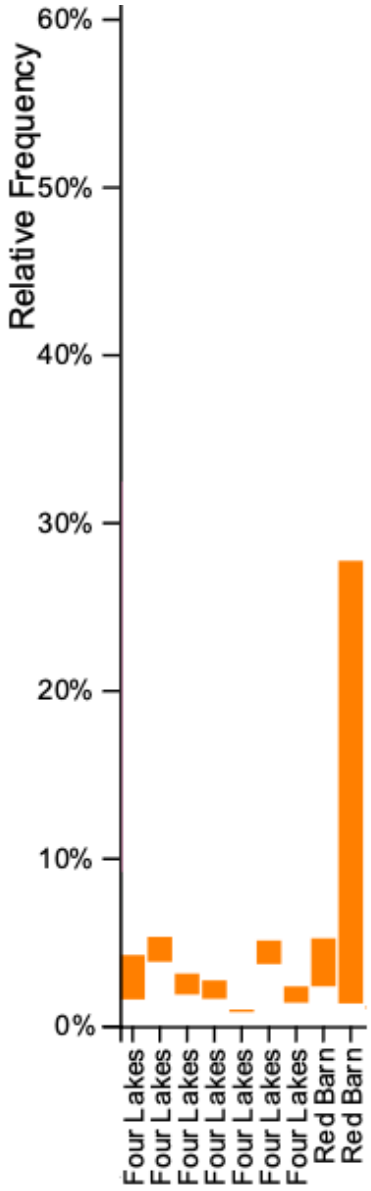

Figure 2.22. Relative abundance bar plot showing relative abundance of *Bartonella* in October across apiary locations. *Bartonella* was higher in Red Barn hives than Four Lake hives at this timepoint (Four Lakes mean = 1.3%, Red Barn mean = 15%, LDA = 4.85, p-value = 0.04).

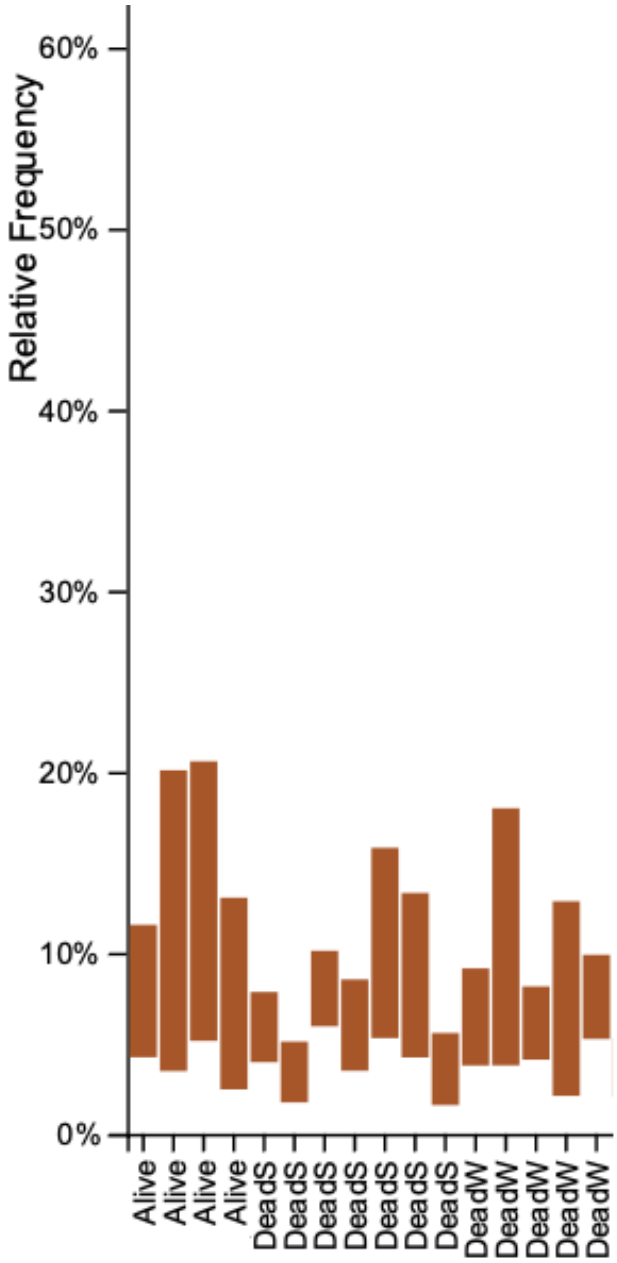

Figure 2.23. Relative abundance bar plot showing the relative abundance of *Snodgrasella* at the April timepoint across survival statuses. *Snodgrasella* was higher in hives that survived the experiment than those that died over summer or over winter (Figure 2.23, Alive mean = 12.5%, Dead Summer mean = 5.7%, Dead Winter mean = 7.8%, LDA = 4.81, p-value = 0.043).

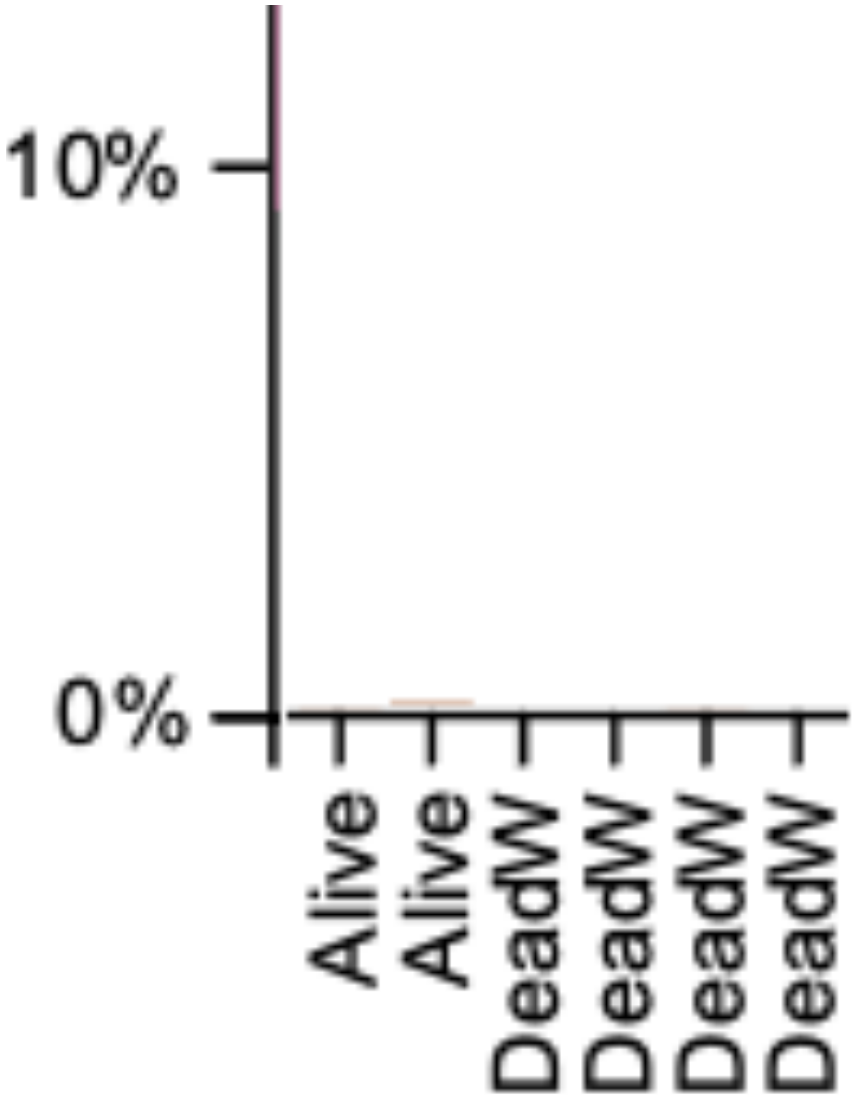

Figure 2.24. Relative abundance bar plot showing the relative abundance of *Providencia* at the September timepoint across survival statuses. *Providencia* was higher in hives that survived the experiment than those lost over winter, although its relative abundance was very low (Alive mean = 0.02%, Dead Winter mean = 0%, LDA = 2.35, p-value = 0.049).

Tables:

Table 1.1 – Number of hives assigned to each treatment type and location.

	Four Lakes	Red Barn	Total:
Nectar	4	4	8
Sugar	4	4	8
Total:	8	8	16

Table 1.2 – Nutrients in synthetic nectar feeding treatment. Nutrients are mixed into a 5-gallon bucket of 1:1 sugar to water.

Nutrient:	Per 5-gal sugar water:
Calcium (Calcium Citrate)	2.7 grams
Magnesium (Magnesium Chloride)	0.9 grams
Phosphorus (Disodium Phosphate)	1.8 grams
Potassium (Potassium Chloride)	23.4 grams
Sodium (Sodium Chloride)	2.25 grams
Zinc (Zinc Citrate)	0.099 grams
Casein	135 grams
p-Coumaric Acid	4.5 grams
Niacin	27.48 milligrams
Pantothenic Acid	15.44 milligrams
Vitamin B6	5.45 milligrams
Folate	0.45 milligrams
Vitamin C	113.55 milligrams
Riboflavin	8.63 milligrams

Table 1.3 – Feeding and sampling schedule.

	April 28, 2018	May 23, 2018	August 23, 2018	September 12, 2018	October 3, 2018
Samples:	Initial hive set up Pre-feeding sample	Post-feeding sample	No treatment sample	Pre-feeding sample	Post-feeding Sample

Table 1.4. This table shows all statistical information for hive weight at individual timepoints by Treatment, Status, and Location. This table also notes what weight timepoints microbiome sampling occurred and the date where the 2nd hive box was added to the growing hives. Highlighted p-values indicate significance.

Timepoints	Date	Treatment (p-value)	Treatment (Chi-Squared)	Status (p-value)	Status (Chi-Squared)	Location (p-value)	Location (Chi-Squared)	Notes
1	28-Apr-18	1	0	0.71	0.68	0.13	2.265	Microbiome Sampling
2	1-May-18	0.08	3.05	0.77	0.53	0.52	0.4	
3	5-May-18	0.75	1	0.28	2.54	0.79	0.07	
4	11-May-18	0.96	0.003	0.53	1.28	0.96	0.0027	
5	18-May-18	0.64	0.22	0.87	0.29	0.49	0.47	
6	23-May-18	0.64	0.22	0.81	0.42	0.79	0.07	
7	23-May-18	0.67	0.18	0.58	1.07	0.17	1.86	Microbiome Sampling
8	25-May-18	0.92	0.11	0.98	0.03	0.4	0.71	Added 2nd Hive Box
9	1-Jun-18	0.75	0.1	0.25	2.76	0.046	3.98	
10	6-Jun-18	0.4	0.7	0.09	4.77	0.016	5.83	
11	12-Jun-18	0.46	0.54	0.17	3.49	0.0087	6.89	
12	1-Jul-18	0.67	0.18	0.36	2.04	0.75	0.099	
13	6-Jul-18	0.92	0.011	0.2	3.2	0.83	0.04	
14	22-Jul-18	0.83	0.04	0.12	4.13	0.29	1.1	
15	23-Aug-18	0.9	0.01	0.02	7.67	0.09	2.82	
16	25-Aug-18	0.33	0.96	0.33	0.96	0.77	0.086	Microbiome Sampling
17	13-Sep-18	0.86	0.03	0.48	0.5	N/A	N/A	Microbiome Sampling
18	28-Sep-18	0.62	0.24	0.81	0.06	0.38	0.77	
19	10-Oct-18	0.62	0.24	0.22	1.5	1	0	Microbiome Sampling
20	18-Mar-19	1	0	N/A	N/A	N/A	N/A	

Table 1.5. Hive Survival Status where “DeadS” are hives that were lost over summer, “DeadW” are hives that were lost over winter, and “Alive” are hives that survived the one-year experiment.

ID	Location	Treatment	Status
1S	Red Barn	Sugar	DeadS
2S	Red Barn	Sugar	DeadS
3S	Red Barn	Sugar	DeadS
4S	Red Barn	Sugar	Alive
5N	Red Barn	Nectar	DeadW
6N	Red Barn	Nectar	DeadS
7N	Red Barn	Nectar	DeadS
8N	Red Barn	Nectar	DeadS
1N	Four Lakes	Nectar	DeadW
2N	Four Lakes	Nectar	DeadW
3N	Four Lakes	Nectar	Alive
4N	Four Lakes	Nectar	Alive
5S	Four Lakes	Sugar	DeadW
6S	Four Lakes	Sugar	DeadW
7S	Four Lakes	Sugar	Alive
8S	Four Lakes	Sugar	DeadS

Table 2.1. Summary of alpha diversity results across all timepoints, alpha diversity metrics, and variables. Highlighted p-values indicate significance. Cells with "N/A" represent when test was not applicable (i.e. only one location sampled) or where sample size was too low for statistical analysis.

Timepoint	Stat	Treatment	Status	Location	TrtStatus	TrtLocation	Weight	Weight Gain
April	Observed OTUs	0.58	0.84	0.37	0.50	0.42	0.87	N/A
April	Evenness	0.75	0.36	0.60	0.80	0.90	0.80	N/A
April	Shannon	0.34	0.58	0.83	0.53	0.81	0.89	N/A
April	Faith's PD	0.67	0.68	0.40	0.86	0.66	0.90	N/A
May	Observed OTUs	0.27	0.018	0.46	0.12	0.49	0.11	0.89
May	Evenness	0.67	0.82	0.35	0.68	0.76	0.57	0.70
May	Shannon	0.83	0.39	0.29	0.71	0.67	0.73	0.80
May	Faith's PD	0.058	0.94	0.75	0.47	0.21	0.96	0.81
August	Observed OTUs	0.62	0.9	0.076	0.80	0.30	0.98	0.93
August	Evenness	0.46	0.8	0.24	0.70	0.48	0.14	0.10
August	Shannon	0.14	0.8	0.77	0.13	0.28	0.067	0.06
August	Faith's PD	0.62	0.33	0.38	0.68	0.80	0.46	0.60
September	Observed OTUs	0.50	0.81	N/A	0.80	N/A	0.007	0.0003
September	Evenness	0.82	0.16	N/A	0.37	N/A	0.20	0.60
September	Shannon	0.83	0.35	N/A	0.73	N/A	0.04	0.20
September	Faith's PD	0.28	0.35	N/A	0.54	N/A	0.40	0.33
October	Observed OTUs	0.8	0.13	0.37	0.19	0.70	0.63	0.63
October	Evenness	0.81	1	1	0.37	0.44	0.14	0.14
October	Shannon	0.81	0.14	1	0.40	0.17	0.09	0.09
October	Faith's PD	1	0.014	0.38	0.11	0.66	0.33	0.33

Table 2.2. Summary of beta diversity results across all timepoints, beta diversity metrics, and variables. Highlighted p-values indicate significance. During August and October, the location and combination of Treatment and Status were excluded due to low sample size. Cells with "N/A" represent when test was not applicable (i.e. only one location sampled) or where sample size was too low for statistical analysis.

Timepoint	Stat	Treatment	Status	Location	TrtStatus	TrtLocation	Weight	Weight Gain
April	Bray-Curtis	0.22	0.025	0.36	0.056	0.24	0.60	N/A
April	Jaccard	0.27	0.17	0.16	0.25	0.27	0.41	N/A
April	Unweighed Unifrac	0.39	0.67	0.07	0.84	0.34	0.44	N/A
April	Weighted Unifrac	0.97	0.27	0.51	0.63	0.958	0.38	N/A
May	Bray-Curtis	0.63	0.19	0.12	0.53	0.615	0.38	0.57
May	Jaccard	0.15	0.047	0.43	0.006	0.108	0.27	0.55
May	Unweighed Unifrac	0.44	0.42	0.36	0.68	0.256	0.15	0.77
May	Weighted Unifrac	0.60	0.32	0.31	0.60	0.512	0.09	0.89
August	Bray-Curtis	0.65	0.87	0.17	0.78	N/A	0.78	0.89
August	Jaccard	0.40	0.044	0.38	0.12	N/A	0.45	0.42
August	Unweighed Unifrac	0.13	0.83	0.25	0.09	N/A	0.69	0.55
August	Weighted Unifrac	0.26	0.91	0.035	0.19	N/A	0.83	0.71
September	Bray-Curtis	1	0.33	N/A	N/A	N/A	0.97	0.96
September	Jaccard	0.67	1	N/A	N/A	N/A	0.33	0.55
September	Unweighed Unifrac	0.61	0.67	N/A	N/A	N/A	0.76	0.67
September	Weighted Unifrac	0.79	1	N/A	N/A	N/A	0.73	0.77
October	Bray-Curtis	0.20	0.96	0.048	0.69	N/A	0.096	0.24
October	Jaccard	0.42	0.31	0.12	0.30	N/A	0.66	0.87
October	Unweighed Unifrac	0.12	0.023	0.51	0.004	N/A	0.28	0.24
October	Weighted Unifrac	0.26	0.84	0.064	0.68	N/A	0.56	0.64

APPENDIX

RStudio – Analyzing Weight at Individual Timepoints

1. Import data

```
weight_timepoint_X <- read.csv(file="Weight_TimepointX.csv", header=TRUE)
```

2. Kruskal Wallis Statistical Test; Weight versus each variable (Treatment, Location, Survival Status)

```
kruskal.test(Weight ~ Location, data=weight_timepoint_X)
```

3. Visualize using Box Plot

```
boxplot(Weight~Location, data=weight_timepoint_X)
```

4. Repeat for each additional timepoint

RStudio – Analyzing Weight Over all Timepoints

1. Open required packages

```
library(nlme)
```

2. Import data

```
weight_all_time <- read.csv(file="WeightR_everything.csv", header=TRUE)
```

3. Linear Mixed Effect Model by Individual Variable (Treatment, Location, Survival Status)

```
m1 <- lme(Weight~Treatment, random=~1|ID, data=weight_all_time, na.action=na.exclude)
```

4. Summarize Model

```
summary(m1)
```

5. Visualize Using Line Graph

```
mean_trt <- read.csv("mean_trt.csv")
```

```
opar <- theme_update(panel.grid.major = element_line(colour="grey85"), panel.grid.minor =
element_line(colour="grey80"), panel.background = element_rect(fill="white", linetype =
"solid", colour = "black"))
gp <- ggplot(mean_trt, aes(x=Timepoint, y=MeanWeight, colour=Treatment,
group=Treatment))
gp + geom_line(aes(linetype=Treatment), size=.6) + geom_point(aes(shape=Treatment), size=3)
+  geom_errorbar(aes(ymax=MeanWeight+StandardError, ymin=MeanWeight-StandardError),
width=.1) + ylab ("Weight (kg)") + ggtitle("Hive Weight Over Time by Treatment") +
theme(plot.title = element_text(hjust = 0.5))
theme_set(opar)
```

RStudio – Analyzing Mite Counts

1. Import data – done twice, once for each mite sampling timepoint

```
mites_april<-read.csv(file="Mites_April.csv", header = TRUE)
```

2. Run two-tailed t-test against variables (Treatment, Location, Status)

```
t.test(Mites~Treatment, data=mites_april)
```

3. Visualize using boxplot

```
boxplot(Mites~Treatment, data=mites_april)
```

RStudio – Analyzing Hive Survival Rates

1. Import data

```
survival <- read.csv("weight_timepoint1.csv")
```

2. Create data frame - Done for each variable (Treatment, Location, Status)

```
status.data <- data.frame(survival$Status,survival$Location)
status.data = table(survival$Status,survival$Location)
```

3. Run Fisher's Exact Test

fisher.test(status.data)

Protocol: Dissection and DNA extraction of honeybee gut using Qiagen DNeasy Kit
Based off Koch and Schmid-Hempel 2011 Microbial Ecology [Updated June 2017]

Reagents:

- 5% bleach solution and sterile water for surface sterilization of bees
- Sterile molecular water for DNA elution
- Lysis buffer
- Lysozyme (powder)
- Proteinase K (DNeasy kit)
- Buffer AL, AW1, AW2 (DNeasy kit)
    - If this is the first time you are using the kit, make sure you add ethanol to the appropriate buffers as described in the manufacturer's instructions.

Preparing lysis buffer:

- Prepare and autoclave stock solutions of
    - Tris-HCl pH 8 1M (calibrate pH with HCl)
    - EDTA pH 8 0.5M (calibrate pH with NaOH pellets)

- Prepare and autoclave lysis buffer
    - 20mM Tris-HCl pH 8
    - 2mM EDTA pH 8
    - 1.2% Triton-x-100

Preparing lysing solution: (do this immediately prior to sample collection)

- Measure out lysozyme into a sterile falcon tube; sterilize spatula (ethanol and flame) before transferring powder. Add appropriate amount of buffer (20mg lysozyme per 1ml lysis buffer). Vortex falcon tube.
    - For 12 samples, make enough for 13 tubes by adding 48 mg lysozyme to 2.4 ml lysis buffer.
    - For 24 samples, 90 mg lysozyme + 4.5 ml lysis buffer.
    - For 30 samples, 111.6 mg lysozyme + 5.58 ml lysis buffer.

Prior to extraction:

- Sterilize workspace, dissection tools, and pipettors with 10% bleach solution.
- Prepare sterile 1.5ml tubes with 180 ul of lysis buffer and lysozyme solution.

Dissection

- To surface sterilize, soak whole bee in 5% bleach solution for 30 sec., followed by three 5 sec. rinses in sterile water (use fresh 1ml aliquots in 1.5ml tubes of bleach and water for each bee). Place bee onto sterile petri dish under dissecting microscope.
- Remove gut from bee by:

    - Pulling off stinger with GI tract attached. Cut out midgut if interested in this section.
    - If necessary, remove abdomen, cut along sides with micro-scissors, remove ventral cuticle, remove whole or midgut.
    - **Flame or bleach sterilize tools between each sample.
- Transfer gut(s) to 1.5ml tube containing 180ul of lysis solution (with lysozyme).
    - Pool 5 bee guts per hive sample

DNA Extraction

- Set heat block to 37°C.
- Grind gut(s) in lysis solution with sterile pestle and mixer for 5 seconds to homogenize solution.
- Incubate at 37C for 1 hour
- Reset thermal block to 56 C.
    - Add 25 ul proteinase k to each tube.
    - Add 200 ul buffer AL.
    - Vortex each sample.
- Incubate at 56 C for 30 minutes.
    - While waiting, set up and label filter/collection tubes (from DNeasy kit) and sterilized storage tubes. Place in racks that have been cleaned and bleached.
- Turn off incubator.
    - Add 200 ul cold ethanol (100%; maintain in freezer) to each tube.
        - Ethanol binds to the DNA and prevents it from washing through the filter.
    - Vortex 5-10 seconds.
    - For pools: Centrifuge at 13,000rpm for 2 minutes, transfer supernatant to spin column (next step).
- Pipette solution into DNeasy mini spin columns (the special filter tubes) that have been placed in the 2 ml collection tubes. Use a large pipette set at ~800 ul. Discard pipette tip every time in between tubes.
    - Centrifuge at 8500 rpm for 1 min.
    - Discard the liquid in the collection tube, along with the tube. Retain the filter tube.
- Place mini spin column in a new collection tube.
    - Add 500 ul AW1 buffer.
    - Centrifuge at 8500 rpm for 1 min; discard collection tube and liquid.
- Place mini spin column in a new collection tube.
    - Add 500 ul AW2 buffer.
    - Centrifuge at 14000 rpm for 3 mins; discard liquid. Centrifuge again for 1 min. then discard liquid and collection tube, being careful not to splash liquid up onto filter.
- Place mini spin column in a clean, sterile 1.5ml storage tube.
    - Pipette 200 ul of sterile water directly onto membrane in tube. Discard pipette tip every time in between tubes.
    - Let sit incubating at room temperature for 5 minutes.
    - Centrifuge at 8500 rpm for 1 min. Be sure to position vials so caps don't break.
- Store DNA at -20 or -80 for long term.

Illumina MiSeq Sequencing Protocol

Adapted from the Earth Microbiome Project
Edited in August, 2017 by: Jeni Walke, Angie Estrada, Daniel Medina, Jessica Hernandez and Lisa Belden. Edited Nov-April 2018-2019 by: Shelby Fettig, Jeni Walke

Reagents:

- UltraClean PCR grade H2O
- 5 Prime Hot Master Mix
- Forward primer + barcode IL 515F
- Reverse primer IL 926R

Before beginning:

- Sterilize workspace with Thermo Scientific RNase AWAY. If possible, perform in a hood dedicated to PCR set up. UV hood before using; UV hood space 15 minutes and open PCR tubes for additional 15 minutes.
- Sterilize pipettors (use pipettors dedicated for PCR reagents and use a separate pipettor for the DNA) with RNAase AWAY.
- Clean and sterilize with 5% bleach: 1 large centrifuge tube rack and several small PCR tube racks. Rinse and allow to dry.
- Locate samples and barcodes. Assign samples to barcodes. Keep both in fridge until ready to use.
- Due to high concentration of DNA, DNA was further diluted using 45 ul of molecule grade PCR water and 5 ul of DNA to create a 1:10 dilution ratio.

Step 1: Make your PCR reactions

- For each sample, you will run triplicate PCR reactions plus a negative control = 4 PCR tubes per sample.

Per sample	4x (4 per sample – triplicate + neg control)
12 ul UltraClean PCR grade H2O	48 ul
10 ul Quantabio 5’ Prime Hot Master Mix	40 ul
0.5 ul Forward primer + barcode IL 515	2 ul
0.5 ul Reverse primer IL 926R	2 ul
23 ul Total (Before DNA)	

2.0 ul DNA 6.0 ul (in triplicate) = 25 ul rxn

1. Set up four rows of PCR tubes. The front row is where we will set up our M^3. The second row will be our negative control. The last two rows will be our second and third replicate of samples.

2. Add all reagents, **except DNA**, to each PCR tube in the first row of the plate.
    a. Each tube will have its own forward primer with assigned barcode; add forward primer last to avoid changing tips between each tube for all other reagents.
    b. Label each tube by barcode.
    c. Vortex gently and briefly centrifuge after all reagents except DNA have been added to all tubes in front row.

3. Pipette 23 ul from the first row of PCR tubes, with every reagent listed above except DNA, into the negative PCR tubes. Label each tube as negative control and barcode number.

4. Add DNA (6ul) to first row.
    a. Vortex gently and centrifuge briefly

5. Take 25 ul from the first row of PCR tubes and add into replicate rows #2 and #3. Label tubes in second and third row with barcode number.

6. Vortex gently and centrifuge each PCR tube, including negative control strip, briefly.

<u>Step 2: Run reactions in thermocycler</u>
1. Make sure machine is set for 25 ul samples.

2. Thermocycler conditions:

Temp	Time	
1. 94°C	3 min	
2. 94°C	45 sec	Denaturing
3. 50°C	1 min	Annealing
4. 72°C	1.5 min	Extension
o Repeat steps 2-4 34x		
5. 72°C	10 min	
6. 4°C	hold	

You can maintain your PCR product in the fridge overnight if you need to wait until the next day to run your gel.

<u>Step 3. Run gels to check amplification and negative controls</u>
1. Combine your three separate PCR reactions into a single PCR tube. Use post-PCR pipettors and tips.

2. Make a 1.5% gel. Combine 140 mL 1X TBE and 2.1 g agarose in a small Erlenmeyer flask. Microwave until just boiling. Swirl. Continue boiling/swirling until solution is clear.

3. Once the solution has cooled slightly, add 14 ul gel red stain.

- Note: Gel red is the dye that stains your DNA for visualization.
- Note: Gel red stain is light sensitive--keep away from light as much as possible.

4. Pour gel into mold and allow to cool completely.

5. On a strip of parafilm, combine 5 ul PCR product and 1 ul loading dye. Pipette up and down to combine.

- Note: Loading dye is the dye that is used to view how far your samples have traveled in the gel during electrophoresis.

6. Reset pipettor to 6 ul. Pipette each sample into gel well.

- As the amount of solution decreases (due to evaporation), you may need to reset your pipette ul setting. Avoid air bubbles in the pipette tip as this will cause the DNA to leak out. Gently pipette solution into wells.

7. Load 5 ul of DNA ladder into gel. You can use a broad range 50-10,000 bp ladder.

8. Run gel at a voltage of ~160 for approximately 20 minutes, until dye is about halfway across gel and each of the three colored bands has separated.

9. Visualize gels. Bands for this primer set will be at ~ 300-350 bp. Sample bands may be a little smeary, but there should not be multiple bands. No bands should be visible for the negative controls.

10. Store PCR products at -20 C until you've accumulated all of the samples that you are going to run on a single Illumina plate before moving on to Step 4.

<u>Step 4: Quantifying the DNA</u>
We use a Qubit 2.0 Florometer and the dsDNA High Sensitivity assay kit. Readings can be a bit fickle, so it is better to do all of your samples on the same day at the same time with the same working solution and standards. This can be done on the countertop. Use post-PCR pipettors and tips.

Before beginning:

- Organize your samples in a single PCR tube rack on ice.
- Label florometry tubes supplied by Qubit in a tube rack with sample names in the same order as they occur in the PCR tube rack.

1. Combine in a 50 ml falcon tube:
Per sample (so multiply by the number of samples you are quantifying, plus your 2 standards, plus a little extra for pipetting)

- 1 ul Qubit reagent

- 199 ul Qubit buffer

Vortex. This is your working solution.

2. Make your standards. Combine 10 ul of each standard with 190 ul working solution. Make a separate solution for each standard and combine in the tubes supplied by Qubit.

3. For your samples: Combine 2-5 ul sample with 198-195 ul working solution. Total solution volume should be 200 ul. Make a separate solution for each sample and combine in the florometry tubes that you labeled already. To get the most accurate measurements, it is very important that you get the precise amount of your entire sample into the working solution. Try 2 ul of sample first. If the readings are too low (there's too little DNA), then redo, increasing the amount sample.

4. Vortex and briefly centrifuge all tubes. Drops of liquid stuck on the sides or lids of tubes can mess up the readings.

5. Incubate at room temperature for 2 min.

6. Read tubes in the Florometer. Specify the amount of sample you used (i.e., 2-5 ul). Record reading in ng/ul.

Step 5: Combine equal amounts of amplicons into a single tube

1. Based on the concentration determined by the Florometer, determine how much of each sample you need to add. The goal is to add the same amount of ng of DNA per sample (~180 ng) into a single, 1.5 ml centrifuge tube.
Example: If Sample 1 has a concentration of 38 ng/ul, you should add 200/38 = 5.3 ul to the pool.

2. Add the appropriate volume of each sample to a single centrifuge tube. This is your pooled sample. Compute the volume of the pooled sample.

Step 6: Clean up pooled sample.

We use the Qiagen QIAquick PCR Clean Up Kit.
If this is the first time you are using the kit, make sure you add ethanol and the PH indicator to the appropriate buffers as described in the manufacturer's instructions.

1. Vortex the pooled sample to thoroughly mix it. Pipette 100 ul of the pooled sampled into a new, clean 1.5 ml centrifuge tube. **Store the remaining, uncleaned pooled sample in storage box in -20C.

2. Add 500 ul of Buffer PB to the 100 ul of your pooled sample. Vortex. Check that the color of the mixture is yellow. If the color of the mixture is orange or violet, add 10 µl of 3 M sodium acetate, pH 5.0, and mix. The color of the mixture will turn to yellow.

3. Place a Qiaquick spin column in a provided 2 ml collection tube.

4. To bind DNA, apply the sample to the QIAquick column and centrifuge for 30–60 s at 13,000 rpm.

5. Discard flow-through. Place the QIAquick column back into the same tube.

6. Wash the pooled sample. Add 0.75 ml Buffer PE to the QIAquick column, let the buffer sit on the filter for 2 min, then centrifuge for 30–60 s at 13,000 rpm.

7. Discard flow-through and place the QIAquick column back in the same tube. Centrifuge the column for an additional 1 min at 13,000 rpm.

8. Place the QIAquick column in a new, clean 1.5 ml centrifuge tube.

9. To elute the DNA, add 50 ul water to the QIAquick column, let the buffer sit on the filter for 3 min, then centrifuge for 1 min at 13,000 rpm.

10. Measure the concentration of the cleaned, pooled sample using the Qubit Florometer (as above, but with only one sample) and the 260/280 using the Nanodrop. 260/280 should be between 1.8-2.0.

Step 7: Add PhiX

For running these libraries in the MISeq and HiSeq, you may need to make your sample more complex by adding 30-50% PhiX to your run.

However, the sequencing facility may add PhiX for you. Check with the particular sequencing facility you are using for information about adding PhiX. The sequencing facility that we use (listed below) adds PhiX for you.

Step 8: Send for sequencing!

Keep cleaned, pooled sample frozen until ready to send. Send sample on dry ice.

Sequencing Facility and contact info:

Zach Herbert <zherbert@research.dfci.harvard.edu>
Molecular Biology Core Facilities
Dana Farber Cancer Institute at Harvard
http://mbcf.dfci.harvard.edu/

Nosema PCR Protocol

Adapted from the Earth Microbiome Project
Edited in August, 2017 by: Jeni Walke, Angie Estrada, Daniel Medina, Jessica Hernandez and Lisa Belden. Edited Nov-April 2018-2019 by: Shelby Fettig, Jeni Walke; Edited Oct 2019 based off Illumina MiSeq Protocol

Reagents:

- UltraClean PCR grade H2O
- 5 Prime Hot Master Mix
- Forward primer
- Reverse primer
- *Nosema* positive control

Before beginning:

- Sterilize workspace with RNA away. If possible, perform in a hood dedicated to PCR set up. UV hood before using; UV hood space 15 minutes and open PCR tubes for additional 15 minutes.
- Sterilize pipettors (**use pipettors dedicated for PCR** reagents and use a separate pipettor for the DNA) with bleach and ethanol or with RNA away.
- Clean and sterilize with 5% bleach: 1 large centrifuge tube rack and several small PCR tube racks. Rinse and allow to dry.
- Locate samples and reagents. Keep both in fridge until ready to use.

Step 1: Make your PCR reactions

- For each sample, you will run one PCR reaction.
- You will run one negative control with no DNA and one positive control with *Nosema* DNA each run.
- For samples that might have LOW DNA CONCENTRATIONS, the PCR reactions could be prepared with the same method as below, but with a small change in the volume of the reagents and DNA; additionally BSA could be added to increase PCR yield.

Per sample	M^3 = 8x (# of samples + extra for pipetting)
2.6 ul UltraClean PCR grade H2O	20.8 ul
0.4 ul $MgCl_3$	40 ul
5 ul 5 Prime Hot Master Mix	3.2 ul
0.4 ul Forward primer Nos GenF	4 ul

0.4 ul Reverse primer Nos GenR 4 ul

9 ul Total (Before DNA)

+ 1 ul DNA

3. Add all reagents **EXCEPT DNA** into a 1.5 mL centrifuge tube. This is your M^3.

4. Pipette 9 ul of M^3 into each of your sample PCR tubes.

3. Add DNA (1ul) to each tube EXCEPT the negative control.

4. Vortex gently and centrifuge each PCR tube, including negative control strip, briefly.

Step 2: Run reactions in thermocycler
1. Make sure machine is set for 10 ul samples.

2. Thermocycler conditions:

Temp	Time	
7. 94°C	2 min	
8. 94°C	30 sec	Denaturing
9. 55°C	30 sec	Annealing
10. 72°C	1.5 min	Extension
o	Repeat steps 2-4 34x	
11. 72°C	10 min	
12. 4°C	hold	

You can maintain your PCR product in the fridge overnight if you need to wait until the next day to run your gel.

Step 3. Run gels to check amplification and negative controls

1. Make a 1.5% gel. Combine 140 mL 1X TBE and 2.1 g agarose in a small Erlenmeyer flask. Microwave until just boiling. Swirl. Continue boiling/swirling until solution is clear.

2. Once the solution has cooled slightly, add 14 ul gel red stain.

3. Pour gel into mold and allow to cool completely.

4. On a strip of parafilm, combine 5 ul PCR product and 1 ul loading dye. Pipette up and down to combine.

> Note: loading dye is the dye that is used to view how far your samples have traveled in the gel during electrophoresis.

5. Reset pipettor to 6 ul. Pipette each sample into gel well.

As the amount of solution decreases (due to evaporation), you may need to reset your pipette ul setting. Avoid air bubbles in the pipette tip as this will cause the DNA to leak out. Gently pipette solution into wells.

6. Load 5 ul of DNA ladder into gel. You can use a broad range 50-10,000 bp ladder.

7. Run gel at a voltage of ~160 for approximately 20 minutes, until dye is about halfway across gel and each of the three colored bands has separated.

8. Visualize gels. Bands for this primer set will be between 1200 and 1500 bp when comparing to DNA ladder. Sample bands may be a little smeary, but there should not be multiple bands. No bands should be visible for the negative controls and a strong band visible for the positive control.

QIIME Bioinformatics Processing Protocol

Installing QIIME2

1. Install Miniconda
    a. https://docs.conda.io/en/latest/miniconda.html
    b. https://conda.io/projects/conda/en/latest/user-guide/install/macos.html
    c. You can choose python 2 or 3; QIIME will work with either
2. Install QIIME 2 w/in a conda environment
    a. Run the following code, one line at a time
        i. wget https://data.qiime2.org/distro/core/qiime2-2019.1-py36-osx-conda.yml
        ii. conda env create -n qiime2-2019.1 --file qiime2-2019.1-py36-osx-conda.yml
3. Activate conda environment
    a. Conda activate qiime2-2019.1
4. Test installation
    a. Run command:
        i. Qiime –help

Importing Sequence Data

- Fastq files were downloaded from the sequencing facilities' website (https://distrib.dfci.harvard.edu). The sequencing facility already demultiplexed the files, meaning there were individual fastq files for each forward and reverse sample sequence.
- Files were imported into QIIME using a manifest table that contained the location of each sequence file, whether it was a forward or reverse sample sequence, and the sample ID.
    - qiime tools import \
    - --type 'SampleData[PairedEndSequencesWithQuality]' \
    - --input-path Manifest.csv \
    - --input-format PairedEndFastqManifestPhred33 \
    - --output-path paired-end_demux.qza

Checking Sequence Quality Scores

- qiime demux summarize \

  --i-data paired-end_demux.qza \

  --o-visualization demux.qzv
- Any files ending in "qzv" are visualized at view.qiime2.org

Filtering Low Quality Scores and Trimming Sequences

- qiime dada2 denoise-paired \

```
--i-demultiplexed-seqs paired-end_demux.qza \
--p-trim-left-f 12 \
--p-trim-left-r 13 \
--p-trunc-len-f 228 \
--p-trunc-len-r 217 \
--o-representative-sequences rep-seqs-dada2.qza \
--o-table table-dada2.qza
--o-denoising-stats stats-dada2.qza
```

Create Feature Classifier with Silva v132 Database

- Database downloaded from https://www.arb-silva.de/no_cache/download/archive/release_132/Exports/
- Import Database and Taxonomy Files:

```
qiime tools import \
 --type 'FeatureData[Sequence]' \
 --input-path silva_132_99_16s.fna \
 --output-path 85_otus.qza

qiime tools import \
 --type 'FeatureData[Taxonomy]' \
 --input-format HeaderlessTSVTaxonomyFormat \
 --input-path 85_otu_taxonomy.txt \
 --output-path ref-taxonomy.qza
```

- Extract Reads Using 515F 926R Primer Set:

```
qiime feature-classifier extract-reads \
 --i-sequences silva_132_99_16s.qza \
 --p-f-primer GTGYCAGCMGCCGCGGTAA \
 --p-r-primer CCGYCAATTYMTTTRAGTTT \
 --p-min-length 0 \
 --p-max-length 0 \
 --o-reads extractreads_silva_99_16s_ref-seqs.qza
```

- Train Classifier:

```
qiime feature-classifier fit-classifier-naïve-bayes \
--i-reference-reads silva_99_16s_ref-seqs.qza \
--i-reference-taxonomy silva_132_99_16s_taxonomy_all_levels.qza \
--o-classifier silva_99_132_16s_classifer.qza
```

Classify Dataset

- qiime feature-classifier classify-sklearn \
  --i-classifier silva_99_132_16s_classifer.qza \
  --i-reads rep-seqs.qza \
  --o-classification taxonomy.qza

Filter Mitochondria and Chloroplasts

- qiime taxa filter-table \
  --i-table table.qza \
  --i-taxonomy taxonomy.qza \
  --p-exclude mitochondria,chloroplasts \
  --o-filitered-table filtered_table.qza
- qiime taxa filter-seqs \
  --i-sequences rep-seqs.qza \
  --i-taxonomy taxonomy.qza \
  --p-exclude mitochondria,chloroplasts \
  --o-filtered-sequences filtered_rep_seqs.qza

Visualize Feature Table

- qiime feature-table summarize \
  --i-table filtered_table.qza \
  --o-visualization filtered_table.qzv \
  --m-sample-metadata-file metadata.tsv

Create Phylogenetic Tree

- qiime phylogeny align-to-tree-mafft-fasttree \
  --i-sequences filtered_rep-seqs.qza \
  --o-alignment aligned-rep-seqs.qza \
  --o-masked-alignment masked-aligned-rep-seqs.qza \
  --o-tree unrooted-tree.qza \

--o-rooted-tree rooted-tree.qza

Visualize Alpha Rarefaction Plot to Determine Sampling Depth

- qiime diversity alpha-rarefaction \
  --i-table filtered_table.qza \
  --i-phylogeny rooted-tree.qza \
  --p-max-depth 62000 \
  --m-metadata-file metadata.tsv \
  --o-visualization alpha-rarefaction.qzv

Diversity Metrics

- Filter Table to Include a Single Timepoint
  qiime feature-table filter-samples \
  --i-table filtered_table.qza \
  --m-metadata-file metadata.tsv \
  --p-where "Timepoint='April'" \
  --o-filtered-table April-table.qza
    - Repeat for each timepoint
- qiime diversity core-metrics-phylogenetic \
  --i-phylogeny rooted-tree.qza \
  --i-table filtered_table.qza \
  --p-sampling-depth 30834 \
  --m-metadata-file metadata.tsv \
  --o-rarefied-table rare-table.qza
  --output-dir core-metrics-results
    - Output folder will contain all alpha metrics (Default: Shannon, observed otus, faith's, evenness) and beta metrics (jaccard, bray-curtis, unweighted unifrac, weighted unifrac).
    - Repeat for each timepoint

Alpha Diversity

- Group Significance (Comparing categorical variables with Kruskal-Wallis)
    - qiime diversity alpha-group-significance \
      --i-alpha-diversity core-metrics-results/faith_pd_vector.qza \
      --m-metadata-file metadata.tsv \
      --o-visualization core-metrics-results/faith-pd-group-significance.qzv
    - Repeat for all alpha diversity metrics and at all timepoints
- Correlation (Comparing numerical variables with Spearman Rank Test)
    - qiime diversity alpha-correlation \
      --i-alpha-diversity core-metrics-results/faith_pd_vector.qza \
      --m-metadata-file metadata.tsv \
      --p-method Spearman
      --o-visualization core-metrics-results/faith-pd-group-significance.qzv
    - Repeat for all alpha diversity metrics and at all timepoints

Beta Diversity

- Group Significance (Comparing categorical variables with PERMANOVA)
    - qiime diversity beta-group-significance \
      --i-distance-matrix core-metrics-results/unweighted_unifrac_distance_matrix.qza \
      --m-metadata-file metadata.tsv \
      --m-metadata-column Treatment \
      --o-visualization core-metrics-results/unweighted-unifrac-treatment-significance.qzv \
      --p-pairwise
    - Repeated for all categorical variables and all diversity metrics at all timepoints
- Correlation (Comparing numerical variables using Mantel test)
    - qiime diversity beta-correlation \

--i-distance-matrix core-metrics-
results/unweighted_unifrac_distance_matrix.qza \
--m-metadata-file metadata.tsv \
--m-metadata-column Weight \
--o-metadata-distance-matrix filename.qza \
--o-mantel-scatter-visualization filename.qzv
--p-intersect-ids

    - Repeated for all numerical variables and all diversity metrics at all timepoints
- Visualize with Emperor Plot
    - Used to visualize beta diversity distance metrics with interactive PCoA
    - Part of initial core-metrics-phylogeny output

Relative Abundance

- Visualize Microbiome with Taxa Bar Plot

  qiime taxa barplot \
  --i-table filtered_table.qza \
  --i-taxonomy taxonomy.qza \
  --m-metadata-file metadata.tsv \
  --o-visualization taxa-bar-plots.qzv

- Collapse Table to Level 6 (Genus)

  qiime taxa collapse \
  --i-table table.qza \
  --i-taxonomy taxonomy.qza \
  --p-level 6 \
  --o-collapsed-table table-l6.qza

- Export Feature Table to biom file for LEfSe analysis

qiime tools export \

--input-path table-l6.qza \

--output-path table-l6

- Convert biom file to tsv

  biom convert \

  --input-fp table-l6.biom \

  --output-fp table-l6.txt \

  --header-key “taxonomy” --to-tsv

- Divide output tsv file into five separate files for each timepoint by cutting all samples at the particular timepoint into a new excel workbook
- Edit text file in excel
  - Remove “#Constructed from biom file” line
  - Add variable to top row
  - Replace “#OTU ID” with “SampleID”
  - Repeat for each variable (Treatment, Location, Status) at each timepoint
- Upload file to http://huttenhower.sph.harvard.edu/galaxy/
  - Follow instructions for steps 1-4. Default setting were used.

PICRUSt Protocol

- Install package

  wget https://github.com/gavinmdouglas/q2-picrust2/releases/download/v0.0.2/q2-picrust2-0.0.2.zip \

  unzip q2-picrust2-0.0.2.zip \

  cd q2-picrust2-0.0.2 \

  python setup.py install \

  qiime dev refresh-cache

- Run pipeline

  qiime picrust2 full-pipeline

  --i-table mammal_biom.qza \

  --i-seq mammal_seqs.qza \

  --output-dir q2-picrust2_output \

  --p-threads 1 \

  --p-hsp-method pic \

  --p-max-nsti 2

  –verbose

- Visualize feature table

  qiime feature-table summarize

  --i-table q2-picrust2_output/pathway_abundance.qza

  --o-visualization q2-picrust2_output/pathway_abundance.qzv

- Export for analysis in RStudio

  qiime tools export \

  --input-path q2-picrust2_output/pathway_abundance.qza \

  --output-path pathabun_exported

  biom convert \

  -i pathabun_exported/feature-table.biom \

  -o pathabun_exported/feature-table.biom.tsv \

  --to-tsv

- Categorize using RStudio

```
categorize_by_function_l3 <- function(in_ko, kegg_brite_mapping) {
 out_pathway <- data.frame(matrix(NA, nrow=0, ncol=(ncol(in_ko) + 1)))
 colnames(out_pathway) <- c("pathway", colnames(in_ko))
 for(ko in rownames(in_ko)) {
  if(! ko %in% rownames(kegg_brite_mapping)) {
   next
  }
  pathway_list <- strsplit(kegg_brite_mapping[ko, "metadata_KEGG_Pathways"],
"\\|")[[1]]
  for(pathway in pathway_list) {
   pathway <- strsplit(pathway, ";")[[1]][3]
   new_row <- data.frame(matrix(c(NA, as.numeric(in_ko[ko,])), nrow=1,
ncol=ncol(out_pathway)))
   colnames(new_row) <- colnames(out_pathway)
   new_row$pathway <- pathway
   out_pathway <- rbind(out_pathway, new_row)
  }
 }
 out_pathway = data.frame(aggregate(. ~ pathway, data = out_pathway, FUN=sum))
 rownames(out_pathway) <- out_pathway$pathway
 out_pathway <- out_pathway[, -which(colnames(out_pathway) == "pathway")]
 if(length(which(rowSums(out_pathway) == 0)) > 0) {
  out_pathway <- out_pathway[-which(rowSums(out_pathway) == 0), ]
 }
 return(out_pathway)
}
kegg_brite_map <- read.table("picrust1_KO_BRITE_map.tsv",
                     header=TRUE, sep="\t", quote = "", stringsAsFactors = FALSE,
comment.char="", row.names=1)
```

```
test_ko <- read.table("picrust_alltimepoints.tsv", header=TRUE, sep="\t", row.names=1)
test_ko_L3 <- categorize_by_function_l3(test_ko, kegg_brite_map)
test_ko_L3_sorted <- test_ko_L3[rownames(test_ko_L3), ]
write.csv(test_ko_L3_sorted, "alltime_ko_l3_sorted.csv")
```

- Import data table and metadata file into STAMP
- Sort by timepoint
- Set variable of interested (treatment, location, and survival status)
- Set statistical test
    - For multiple groups, choose Kruskal-Wallis with Bonferroni p-value correction and Games-Howell post-hoc test
    - For two groups, choose White's Non-Parametric t-test with bootstrap confidence interval
- Run statistical analysis and view results

VITA

AUTHOR: SHELBY P. FETTIG

RESEARCH INTERESTS

Microbial and disease ecology: I'm interested in examining the relationship between disease and the microbiome of hosts, specifically in organisms threatened by environmental or climate changes, using a multidisciplinary approach.

EDUCATION

Eastern Washington University — Cheney, WA
Master of Science, Biology (expected 2021) — **2018-2021**
Area of Concentration: Microbiology
Advisor: Dr. Jenifer Walke
GPA: 3.97/4.0

Eastern Washington University — Cheney, WA
Bachelor of Science, Biology, *Cum laude* — **2016-2018**
Area of Concentration: Microbiology
Advisor: Dr. Jenifer Walke
Minor: Spanish

RESEARCH EXPERIENCE

Eastern Washington University
Graduate Research Assistant – Walke Lab — **2018 – 2020**
Master's thesis project: "Effect of Nutrition on Honey Bee Gut Microbiology, Disease Occurrence, and Hive Growth"

- Explored how a lab-created nutritional supplementation meant to mimic natural plant pollen and nectar affected several factors of honey bee health, including gut microbiome structure and function, susceptibility to diseases like *Nosema* and *Varroa* mites, hive growth and productivity, and hive survival
- Assisted with setting up campus apiary and maintained bee hives

"Effect of Pesticide Exposure on the Honey Bee Gut Microbiome", Fall 2017 Pesticide Survey (cont. from undergraduate project):

- Mentored undergraduate directed study student with analyzing honey bee gut microbiome DNA sequence data using QIIME v2 to investigate whether pesticide exposure impacts the gut microbiome

"Survey of White Nose Syndrome via *Pseudogymnoascus destructans* and General Health with Disease Progression in Yuma myotis bats (*Myotis yumanensis*) in Lincoln County, Washington", White Nose Survey in Bats (cont. from undergraduate project):

- Returned to roosting site in Eastern Washington with Washington Department of Fish and Wildlife to sample bat skin to analyze skin microbiome via 16s rRNA amplicon sequencing in order to continue survey for White Nose Syndrome (WNS) and explore potential correlations between WNS, ectoparasites, and skin microbiome. Mentored undergraduate students with associated lab and bioinformatics.

Undergraduate Research Assistant — **2017 – 2018**
Honey Bees – Walke Lab

Directed Study: "Improving Honey Bee Colony Health: Assessing the Role of Honey Bee Gut Microbiome in Mediating Pesticide Effects"

- Analyzed honey bee gut microbiome DNA sequence data using QIIME v1 from faculty advisor's project studying the effects of several pesticides on honey bee health and how the addition of probiotics mitigated some of these effects

"Effect of Pesticide Exposure on the Honey Bee Gut Microbiome", Directed Study: Fall 2017 Pesticide Survey

- Assisted with preparing honey bee samples from survey of apiaries across eastern Washington for quantification of pesticide in honey bee body and DNA sequencing of honey bee gut microbiome

Additional Projects:

- Assisted with setting up campus apiary and maintained bee hives
- Assisted with setting up new faculty laboratory, including ordering supplies and organization of equipment

Bats – Magori Lab

Senior Capstone Thesis Project: "Survey of White Nose Syndrome via *Pseudogymnoascus destructans* and General Health with Disease Progression in Yuma myotis bats (*Myotis yumanensis*) in Lincoln County, Washington"

- Sampled bat skin from roosting site in Eastern Washington with Washington Department of Fish and Wildlife and used culture-based approach to analyze skin microbiome

LABORATORY SKILLS

DNA extractions; PCR for next-generation and Sanger sequencing; qPCR; Gel electrophoresis; Making and culturing various types of broths/agars; aseptic technique; identifying unknown cultures; vertebrate and invertebrate dissections; cell staining for microscope visualization; excellent with microscope; experimental design; bioinformatics using python based programs such as QIIME2 (Quantitative Insights Into Microbial Ecology) and PICRUST (Phylogenetic Investigation of Communities by Reconstruction of Unobserved States); data analysis and statistics using R Studio programming.

TEACHING EXPERIENCE

Eastern Washington University

Teaching Assistant:

- All TA positions involved holding office hours and meeting with students upon request, grading lab and course work, implementing lab safety

Introduction to Biology (BIOL 171) **Sept 2018 – Oct 2019**

- Led discussion section for beginning biology students on a variety of topics from experimental design and data analysis to cell division and genetics

Biological Investigations (BIOL 270) **Mar 2018 – June 2018**

- Coached students with developing independent research projects from initial experimental design to data analysis

Microbiology (BIOL 301) and Elementary Microbiology (BIOL 235) **Mar 2017 – June 2019**

- Lectured about lab material
- Assisted with development of new labs
- Maintained live bacterial cultures
- Coached students with hands-on methods such as basic aseptic technique, microscope work, various bacterial staining procedures, culturing using a diverse array of agars and broths
- Developed lab practical

Microbial Ecology Senior Capstone (BIOL 490) **Mar 2019 – June 2019**

- Coached students with developing independent research projects from initial experimental design to data analysis
- Trained students how to preform sterile DNA extractions
- Trained students how to set up PCR for bacterial isolate Sanger sequencing
- Set up multiplexed 96-sample Illumina run for students, including PCR, Qubit for concentration of PCR products, multiplexing samples, and clean-up
    - Samples came from a variety of sources, including bee gut, amphibian skin, bat skin, and soil
- Trained students how to utilize bioinformatics program QIIME v2 for process of sequenced samples

Guest Lecture:

First Year Experience **Mar 2019**

- Led open discussion about importance of honey bees, sustainable food choices, and plant biodiversity
- Tour of campus apiary and community garden

Microbiology (BIOL 301) **Oct 2018**

- 50-minute lecture about metabolic pathways in bacteria

Peer Mentoring:

- Assisted and coordinated graduate, undergraduate, and high school students with research projects

RELATED COURSES *=LABORATORY WORK

Immunology, Gut Microbiology, Research Methods, Current Topics in Molecular Biology and Ecology/Evolution, General Microbiology*, Medical Bacteriology*, Microbial Physiology, Disease Ecology*, Virology, Ecology*, Molecular Biology, Cellular Biology, Organic Chemistry*, Biostatistics, Hematology*, Genetics, Vertebrate Zoology*, Biological Investigations*, Nutrition.

GRANTS

2019

$13,300 Graduate Student Assistantship and tuition waiver for 2019-2020

$750 Research Grant; EWU Department of Biology Graduate Research Grant, “Effect of Nutrition on Honey Bee Gut Microbiome, Disease Occurrence, and Hive Growth”

$250 Travel Grant; EWU Department of Biology Travel Grant to attend ASM Microbe 2019, “Effect of Nutrition on Honey Bee Gut Microbiome, Disease Occurrence, and Hive Growth”

2018

$13,000 Graduate Student Assistantship and tuition waiver for 2018-2019

$750 Research Grant; EWU College of Science, Technology, Engineering, and Mathematics (CSTEM) Undergraduate Research & Creative Activities Fund, “Effect of Overwintering on Honey Bee Gut Microbiome and Hive Survival”

PRESENTATIONS

Eastern Washington University Research and Creative Works Symposium, Cheney, WA **June 2, 2021**

- Oral Presentation, “Effect of Nutrition on Honey Bee Gut Microbiome, Disease Occurrence, and Hive Growth”

American Society for Microbiology, Microbe 2019 Conference, San Francisco, CA **June 23, 2019**

- Poster Presentation, “Effect of Nutrition on Honey Bee Gut Microbiome, Disease Occurrence, and Hive Growth”

West Plains Beekeeper Association, Medical Lake, WA **May 17, 2019**

- Oral Presentation, "Effect of Nutrition on Honey Bee Gut Microbiome, Disease Occurrence, and Hive Growth"

Eastern Washington University Research and Creative Works Symposium, Cheney, WA **May 15, 2019**

- Poster Presentation, "Effect of Nutrition on Honey Bee Gut Microbiome, Disease Occurrence, and Hive Growth"

Spokane Area Microbiology (SAM) Group, Spokane, WA **April 23, 2019**

- Oral Presentation, "Effect of Nutrition on Honey Bee Gut Microbiome, Disease Occurrence, and Hive Growth"

American Society for Microbiology, Northwest Branch, Portland, OR **October 6, 2018**

- Poster Presentation, "Improving Honey Bee Colony Health: Assessing the Role of Honey Bee Gut Microbiome in Mediating Pesticide Effects"

Eastern Washington University Research and Creative Works Symposium, Cheney, WA **May 16, 2018**

- Poster Presentation, "Improving Honey Bee Colony Health: Assessing the Role of Honey Bee Gut Microbiome in Mediating Pesticide Effects"
- Oral Presentation, "Survey of White Nose Syndrome via *Pseudogymnoascus destructans* and General Health with Disease Progression in Yuma myotis bats (*Myotis yumanensis*) in Lincoln County, Washington"

CO-PRESENTATIONS

Pacific Northwest Beekeeping Conference, Cheney, WA **February 2019**

- Dr. J.B. Walke's Oral Presentation, "Honey bee gut microbes and their influence on bee and colony health"

Entomological Society of America annual meeting, Vancouver, B.C. **November 2018**

- Dr. J.B. Walke's Oral Presentation, **"**Environmental factors can alter the honey bee gut microbiome**"**

COMMUNITY OUTREACH

Earth Day, Tabled at EWU campus mall to educate public about honey bees **April 2019**
HappBEE Hour with the Bee Girl at EWU, Planning and Volunteer **February 2019**
Pacific Northwest Beekeeping Conference, Program Design and Event Volunteer **February 2019**
Kids and Bees, Mobius Science Center, Spokane, WA Volunteer **February 2019**
Science Olympiad, Regional and State Tournaments, Volunteer for Microbe Mission **Spring 2018**
SuperStars in STEM, Event for Middle School girls interested in STEM, Volunteer **February 2018**

HONORS/CERTIFICATIONS

Beekeeping Certification – West Plains Beekeeper Association **March 2019**
Dean's List - Eastern Washington University **Fall 2016 – Spring 2018**
Teaching Assistant Certification – TRiO Student Support Services **September 2012**

OTHER LEADERSHIP EXPERIENCE

Silverwood Theme Park – Athol, ID

Food and Beverage Manager **Mar 2014 – Present**

- Supervised hiring, scheduling, and training of a department of 350+ employees and 35 concession stands
- Offered training and constructive feedback on work performance for new and returning front line and leadership team members, including supervisors and area managers
- Taught comprehensive training courses on policy, food safety and preparation, and leadership skills
- Conducted new hire interviews

- Handled difficult situations such as understaffing, disputes, administering disciplinary procedures, and terminating employees
- Assumed ownership over team productivity and managed work flow to exceed quality service and profit
- Addressed customer concerns
- Maintained equipment and comfortable troubleshooting any piece of equipment
- Worked with Revenue Manager to build menu mix, including balancing cost of goods, introducing new menu items, and bidding 500+ items to distributors
- Routinely supported any position within concession stands as needed

Jamba Juice – Honolulu, HI
Shift Lead **Aug 2015 – Jan 2016**

- Trained new team members with positive reinforcement and respect
- Conducted successful cash audits at the beginning and end of each shift
- Prepared cash deposit
- Provided exceptional service to customers
- Memorized 100+ recipes for specialty beverages, bowls, and seasonal offerings
- Increased sales of seasonal beverages with special displays and promotions
- Assisted with restaurant set up and grand opening of second store in Ala Moana Center

TRiO Student Support Services – North Idaho College – Coeur d'Alene, ID
Peer Tutor **Aug 2013 – May 2015**

- Tutor for math, biological sciences, and Spanish for a program that supports students who are financially limited, disabled, or first-generation college students.

MEMBERSHIPS

American Society of Microbiology (ASM) **Apr 2019 - Present**
Student Membership

Biology Graduate Student Organization (BGSO) – Eastern Washington University **Dec 2018 – Dec 2019**
Co-Founder

Women in Science – EWU (WiSE) – Eastern Washington University **Sept 2019 – Present**
Member

Alpha Gamma Delta – Sorority at University of Hawaii at Manoa **Aug 2015 - Present**
Alumnae of Delta Sigma Chapter

Microbiology Club – University of Hawaii at Manoa **Aug 2015 – Jan 2016**
Treasurer

Phi Theta Kappa – Honor's Society at North Idaho College **Aug 2013 – Present**
Alumnae

LANGUAGES

English – Native language
Spanish – Speak, read, and write with basic competence