Microbial Diversity of Two Hawaiian picture wings and their Host Plants; Microbes, Mortality and Epicuticular Hydrocarbons

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Abstract

Although the microbiome influences numerous aspects of organismal biology and fitness, the community characterization and role in animal evolution is largely unknown. Microbial communities in insects can have a wide range of interactions from mutualistic to parasitic and can be important in host-plant adaptation. In *Drosophila*, microbial associations and interactions have been studied for over a century and recently have been shown to influence the fitness and evolution of their host. This study utilizes both traditional culturing methods and high-throughput next generation DNA sequencing to evaluate the bacterial and fungal communities associated with two Hawaiian *Drosophila*, *D. sproati* and *D. ochracea* and their host plants, *Cheirodendron trigynum* and *Freycinetia arborea*. The culture-based and high-throughput DNA sequencing approaches provided different but complementary results. Interestingly, we found some overlap between microbiomes of the Hawaiian *Drosophila* and their host plants. The bacterial family Enterobacteriaceae, as well as a variety of yeasts, consisted of the majority of these shared microbes. This suggests that these microbes may play a role in the fly-host plant interaction.

To evaluate the effect of these microbes on Hawaiian *Drosophila*, we exposed a laboratory population of *D. sproati* to antibacterial and antifungal treatments to determine the effect on *D. sproati* survivorship and epicuticular hydrocarbons. Epicuticular hydrocarbons in *Drosophila* serve a dual function, aiding in desiccation resistance and pheromonal communication. Flies exposed to an antifungal treatment had dramatically reduced survivorship when compared to control flies, while antibacterial treated flies were not affected. This suggests that fungi are essential to *Drosophila* survival while bacteria are not. However, male flies that were exposed to the antibacterial treatment showed significant changes in two major epicuticular hydrocarbons. One of which, 2MeC28, has been shown to act as a contact pheromone in other insect species. This study shows that changes in microbial communities, specifically bacteria, influence epicuticular hydrocarbon production of *D. sproati* which can impact multiple levels of fitness.

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Chapter 1
Introduction to Microbe-Insect Interactions, and the Hawaiian *Drosophila, Drosophila sproati* and *Drosophila ochracea*

Insects harbor diverse microbial communities and interactions can range from mutualistic to parasitic in nature. Although the microbiome influences numerous aspects of organismal biology and fitness, the community characterization and role in animal evolution is largely unknown. In *Drosophila*, microbial associations and interactions have been studied for over a century and recently they have shown examples of microbial influence on fitness and evolution (Anagnostou et al. 2010a; Anagnostou et al. 2010b; Sharon et al. 2010; Blum et al. 2013).

Studies on microbial associations and interactions within the *Drosophila* genus have been primarily focused on yeast. These single-celled fungi are an important source of nutrients and are vectored by *Drosophila* (Morais et al. 1995; Anagnostou et al. 2010a; Hamby et al. 2012). These yeasts can affect *Drosophila* fitness in many ways, ranging from a change in larval development times to aiding in parasitism resistance (Anagnostou et al. 2010b). *Drosophila* species not only show preference for certain yeast species during development, but also have been found to regulate yeast densities and community structure on their natural substrate (Stamps et al. 2012). In contrast to the abundant literature of yeast interactions associated with *Drosophila*, recent studies have begun to investigate bacterial diversity and effect on *Drosophila* evolution. Sharon et al. (2010) showed that bacteria play a role in mating preference by altering the levels of cuticular hydrocarbon sex pheromones. Given that development of mating preference is considered to be an early event in speciation, these results could have immense implications on *Drosophila* evolution. Understanding these insect-microbe interactions in wild populations of *Drosophila,* such as those found in Hawaii, may provide insight to microbial roles in *Drosophila* ecology and evolution.

The Hawaiian Islands are an ideal natural laboratory to study the population divergence and speciation (Carson & Clague 1995). The Hawaiian *Drosophila* are an excellent illustration of adaptive radiation and many modes for their rapid speciation have been suggested such as their complex mating behaviors, sexual selection, the numerous habitat types found in Hawaii, and high host plant specificity (Kaneshiro & Boake 1987). Despite the fact that many of these possible modes of speciation and evolution in Hawaiian *Drosophila* are well documented, little is known about their microbial diversity and more investigations into Hawaiian *Drosophila* and their ecological relationship with these microorganisms should provide insight to their rapid divergence.

Hawaiian *Drosophila*

The Hawaiian *Drosophila* are an excellent example of adaptive radiation and consist of nearly 1,000 species (Kaneshiro & Boake 1987). This large and morphologically diverse radiation originated from what is thought to be the arrival of a single colonist approximately 25 million years ago (Magnacca et al. 2008). These 1,000 species can be categorized into two major lineages: the Hawaiian *Drosophila* and *Scaptomyza* (Kaneshiro & Boake 1987), which can be further divided into 4 distinct groups: *the modified mouth parts*, *antopocerus*, *haleakalae* and the *picture wing* (O'Grady & Kidwell 2002). The picture-wings, a clade of approximately 120 species (Carson 1982, Magnacca and Price 2015), are notable for their relatively charismatic features, possessing large body size, colorful wing patterns, and intricate mating rituals. Extreme isolation, sexual selection resulting in divergent mating behaviors, numerous microhabitats generated by diverse geographic and climatic patterns present within a small region, and high host plant specificity are likely responsible for this diversity (Kaneshiro & Boake 1987).

Hawaiian *Drosophila* are very ecologically diverse utilizing 34 of the 87 families of native flowering plants as well as various ferns, sap fluxes, and even spider eggs for larval breeding hosts. Members of the picture-wing clade are often variable in host plant species, but are largely restricted to rotting barks and stems as substrate type (Magnacca et al. 2008). *Drosophila sproati* and *Drosophila ochracea* are closely related species within the Hawaiian picture-wing clade. Their respective host plants are *Cheirodendron trigynum*, a tall woody dicot tree and *Freycinetia arborea* a fibrous monocot vine. Although these flies are closely related, their host plants are phylogenetically distant. The microbial community likely plays a key role in this host plant preference, and differences in microbial community between host plants may contribute to the high host specificity in Hawaiian *Drosophila*. Both bacteria and fungi mediate decomposition, releasing plant micronutrients and volatiles that can indicate high quality substrates and serve as cues to stimulate egg laying (Barker et al. 1994; Barker et al. 1999). The potential importance of microbiome on the fitness and mating preference in *Drosophila* (Anagnostou et al. 2010a; Anagnostou et al. 2010b; Sharon et al. 2010) also indicates that it is possible the microbial associations and interactions with the Hawaiian *Drosophila* have also played a role in their diverse adaptations and rapid speciation.

Drosophila have been used as a model for genetics, developmental biology, and now host-microbe interactions. An organism's microbiota has immense effect on its host, ranging from influencing fitness traits to altering pheromone composition and mating behavior. Symbiotic microbes can also quickly respond to varying stresses, thus aiding the host organism's ability to adapt to a rapidly changing environment. With recent advancements in DNA sequencing technologies and recent boom in *Drosophila* microbiome research, now more than ever the tools are available to expand and apply these types of studies to natural populations of

Drosophila such as those found in Hawaii and elucidate the roles that symbiotic microorganisms play in their ecology, sexual selection, and evolution.

Chapter 2

Microbial Survey of *Drosophila sproati, Drosophila ochracea, Cheirodendron trigynum, and Freycinetia arborea* Using Culturing and Culture-Independent Metagenomic Techniques

Introduction

Drosophila and Yeasts

Culture based studies in the past provided an insight into yeast diversity of *Drosophila* (Bregon 1982; Ganter 2006). These early studies were conducted by cultivating yeasts associated with *Drosophila* on solid media containing high nutrient concentrations for yeast colonies to thrive such as yeast mold agar (Brummel, Ching, Seroude, Simon, & Benzer) and potato dextrose agar (PDA) (Heed et al. 1976). The yeasts commonly associated with *Drosophila* were members of phylum *Ascomycota* and the family *Saccharomycetaceae* (Phaff et al. 1956; Hamby et al. 2012). More recent research examined yeasts associated with 14 phylogenetically and ecologically diverse *Drosophila* using 454 pyrosequencing of the first two domains of the large ribosomal subunit RNA (26S rRNA). The internal transcribed spacer (ITS) region, a widely accepted DNA barcode marker for Fungi (Schoch et al. 2012), was not used to assess the yeast community of *Drosophila* due to its variability of fragment length. The sequencing technologies used would have created biased results towards the shorter fragments, thus the 26S rRNA was used instead (Chandler et al. 2012). This study reinforced the culture-based findings that *Drosophila* microbiomes consist of a small number of abundant taxa relative to those of mammals, the most common genera being *Candida, Crytococcus, Hanseniaspora, Hanusula, Kloecke, Kluyveromyes, Pichia, Rhodotorula, Saccharomyces, Saccharomycopsis,* and *Torulopsis* (Bregon 1982; Ganter 2006). This study also found that yeast community composition was more similar among species who utilize the same host-plant than among species that are more closely related, indicating that host plant has a greater effect that *Drosophila* species on the yeast community composition (Chandler et al. 2012).

It is well known that *Drosophila* act as vectors for yeast and these yeasts provide much of the adult and larval diet (Morais et al. 1995; Anagnostou et al. 2010a; Hamby et al. 2012). However, recent studies have shown that *Drosophila*–yeast interactions are more complex than previously thought. The specific yeast species and density level present during feeding has drastic effects on life history traits such as survival, biomass, and development time (Anagnostou et al. 2010a). Larvae also exerted a preference for those yeast species which provided the most favorable life history traits (Anagnostou et al. 2010a; Anagnostou et al. 2010b).

These yeasts associated with *Drosophila* also seem to enhance resistance to parasitoids. For example, the wasp *Asobara tabida* is an endoparasitoid of many *Drosophila* species throughout Europe and the Northeastern USA (Van Leimpt & Van Alphen 1981). These parasitoid wasps lay eggs within *Drosophila* pupae which can cause a drastic decrease in subsequent adult fitness (Fellowes et al. 1999). The presence of *Kluyveromyes lactis* and *Saccharomyces cerevisiae* has been found to increase haemocytes within *D. molanogaster* haemolymph, leading to a higher rate of encapsulation of *A. tabida* eggs, and thus elimination of the parasitoid threat (Anagnostou et al. 2010b).

Transmission of yeasts can occur both vertically and horizontally between *Drosophila* generations. Starmer et al. (1988) demonstrated the movement of yeasts between the cactophilic fly *Drosophila buzzatii* through laboratory culture based studies. Vertical transmission occurred through the pupal stage, while horizontal transmission of yeasts was accomplished during courtship and mating. It is valuable to broaden our understanding of yeasts and their association with natural populations of *Drosophila* and their multiple roles in diet and fitness. Since host substrate has been found to have a greater influence on yeast composition over the taxonomic relatedness of flies (Chandler et al. 2012), the Hawaiian *Drosophila* in particular provide an interesting case study to further investigate this interaction due to their high host specificity.

***Drosophila* and Bacteria**

It is currently estimated that approximately 99% of bacteria are unculturable (Amann et al. 1995), but due to recent advancements in molecular techniques such as PCR, Sanger sequencing, metagenomics approaches, and high throughput next generation sequencing, researchers have the ability to uncover an astonishing level of microbial diversity in insect hosts and other natural habitats (Backhed et al. 2005; Boetius 2005; Langenheder et al. 2010; Chandler et al. 2011; Ort et al. 2012).

Chandler et al. (2011) assessed the bacterial communities of 20 populations of *Drosophila* from around the world, spanning many different species and diet types. They also compared natural *Drosophila* populations to laboratory stocks of *D. melanogaster* to assess whether or not laboratory cultures can serve as an accurate model of host-microbe interactions that occur in the wild. They found that *Drosophila* have a taxonomically restricted bacterial microbiome with four bacterial families representing three orders making up 90% of all sequences within their dataset. These include *Enterobacteriales*: *Enterobacteriaceae* (60%), *Rhodospirillales*: *Acetobacteraceae* (9%), and *Lactobacillales*: primarily *Lactobacillaceae* and *Enterococcaceae* (21%) (Chandler et al. 2011).

Several species within *Enterobacteriaceae* are endosymbiotic and required for insect nutrition, defense from parasites, and tolerance of heat stress (Douglas 1998; Montllor et al. 2002; Oliver et al. 2003; Moran et al. 2005) The *Enterobacteriaceae*, represent a large family that includes many animal and plant associated bacteria. They are found as free living associates of many insects, including *D. melanogaster* (Madigan et al. 2010).

The family Acetobacteraceae contains several genera collectively known as the acetic acid bacteria (Madigan et al. 2010). These obligate aerobic microbes thrive on high-energy substrates and are usually limited by nutrients other than their primary carbon source. They are common in sugary, acidic and alcoholic habitats, such as fruits and flowers. Possibly due to these habitat preferences, they are commonly associated with insects that consume sugar rich diets (Crotti et al. 2010).

The presence of *Lactobacillus plantarum* has been shown to increase *D. melanogaster* fitness by protecting the fly from *Serratia marcescens*, a *Drosophila* and nosocomial human pathogen as well as *Pseudomonas aeruginosa* PAO1, another nosocomial human and invertebrate pathogen (Blum et al. 2013). *Lactobacillales* are widespread in the environment, and many are associated with animal hosts and fermenting plants. Lactobacilli are Gram-positive, acidophilic bacteria usually found on nutrient-rich resources (Madigan et al. 2010). *Lactobacillus plantarum* in particular is commonly found in many fermented food products, anaerobic plant matter, and *Drosophila* (Kleerebezem et al. 2003; Sharon et al. 2010; Blum et al. 2013). Sharon et al. (2010) were able to link mating preference in *D. melanogaster* with *L. plantarum*. This was accomplished by first demonstrating a mating preference between flies that were reared on molasses medium and flies that were reared on starch medium. After one generation, a preference was established where “molasses flies” preferred to mate with other molasses flies and “starch flies” preferred to mate with other starch flies. Next, treatment of both groups with antibiotics removed mating preference, suggesting that the fly microbiota was responsible for the phenomenon. This was confirmed by infection experiments with microbiota obtained from the fly media (before antibiotic treatment) as well as with a mixed culture of *Lactobacillus* species and a pure culture of *L. plantarum* isolated from starch flies.

Methods

Site Description

All Hawaiian *Drosophila* and host plant material used in this study were collected from the Upper Waiakea Forest Reserve (N19°34’11.45"; W155°12’44.54”). The sampling location was at an elevation of 975m and is characterized as a montane wet forest. This site was chosen due to all study species being present in one location to control for varying environmental conditions (Figure 1).

Field Collection

Host Plant

Rotting *Cheirodendron trigynum* and *Freycinetia arborea* were collected by cutting small sections of the rotting material and placing them into individual sterile 50mL conical centrifuge tubes to take back to the University of Hawaii at Hilo. All gloves and cutting tools were sterilized with 91% isopropyl alcohol prior to use in the field, and cleaned and sterilized between each usage. Upon returning to the laboratory, samples were either immediately placed on bacterial or fungal specific media for culture studies, or stored in 95% ethanol at -80°C for later molecular analysis.

Drosophila

Flies were collected by sponges and multilure traps (Great Lakes IPM Inc.) that were baited with a banana-yeast mixture and a fermented fungus spray and then hung one to two meters from the ground near patches of *Cheirodendron trigynum* and *Freycinetia arborea* (Figure 2). Flies were aspirated from the sponges, immediately transferred into collection vials and brought back to the University of Hawai’i at Hilo where the flies were identified. The collected flies were either be placed in a one-gallon jar for breeding, immediately placed on bacterial or fungal specific media for culture studies, or stored in 95% ethanol at -80°C for later molecular analysis.

Microbial Culture Survey

Two different types of media were used to culture either fungal or bacterial colonies from each fly and their respective host-plant. Nutrient agar (Difco) was used to culture bacteria, YM agar (Difco) supplemented with 100 mg/L of the broad-spectrum antibiotic chloramphenicol (Sigma) to inhibit bacteria growth and culture yeasts. Rotting plant material was used because past studies have found that the rotting portion of the *Cheirodendron trigynum* and *Freycinetia arborea* bark were the egg laying substrate for these flies (reviewed in Magnacca et al. 2008).

For each of the host plant samples, 1 gram of plant material was weighed and homogenized with 1 mL of sterile water by manual grinding in a mortar and pestle to extract bacteria and yeast from the plant matter. This mixture was then diluted in steps of 1/10 with sterile water. Dilutions 10^{-5} through 10^{-10} were plated separately on both Nutrient agar and YM agar. Isolates were then streak plated to obtain single pure colonies. Isolates taken from Nutrient agar plates were grown in Nutrient broth and stored in sterile glycerol (20% v/v) at -80°C. Isolates taken from YM agar plates were grown in YM broth and stored in sterile glycerol (25% v/v) at -80°C.

Each individual fly that was caught using a sterile multilure was allowed to walk on YM agar plates supplemented with 100 mg/L of chloramphenicol for one hour. After one hour, the flies were placed in 10mL of sterile water and vortexed briefly. While leaving the fly in the 10mL of water, 1 mL was then diluted in steps of 1/10 with sterile water. Dilutions 10^{-5} through

10^{-10} were plated separately on YM agar supplemented with 100 mg/L chloramplenicol. Isolates were then streak plated to obtain single pure colonies. Each pure isolate was grown in YM broth and stored in sterile glycerol (25% v/v) at -80°C.

Total genomic DNA from isolated pure colonies on agar plates was extracted using an UltraClean Microbial DNA isolation kit (MO BIO) according to manufacturer's instructions. Bacterial colonies originally isolated from nutrient agar were identified by sequencing their 16S rRNA gene with primers primers 27f (5'-AGA GTT TGA TCC TGG CTC AG-3') and 1492r (5'-GGT TAC CTT GTT ACG ACT T-3') (Lane 1991), and isolated pure fungal colonies from YM agar were identified by sequencing their 26S rRNA with primers NL-1 primer (5'-GCA TAT CAA TAA GCG GAG GAA AAG-3'), NL-4 primer (5'-GGT CCG TGT TTC AAG ACG G) (Kurtzman and Robnett 1997) for species level identification. DNA sequencing was done through the Genomics Core Facility at the University of Hawaii at Hilo using the 3500 Genetic Analyzer (Applied Biosystems). Sequences were then analyzed through NCBI Nucleotide BLAST under default settings and the taxa identified to lowest possible taxonomic level possible from the database.

Metagenomics

Host Plant

Rotting plant samples of *Cheirodendron trigynum* and *Freycinetia arborea* were used for metagenomic analysis of *Drosophila* host plant bacteria communities. Per host plant sample, 1 gram of rotting plant material was flash frozen with liquid nitrogen and homogenized by manual grinding in a mortar and pestle. Microbial DNA was extracted using a Powersoil DNA isolation kit (MO BIO) according to manufacturer's instructions.

The V6 region of the 16S rRNA gene was amplified by fusion method constructed primers. PCR conditions were as follows: initial denaturation for 3 minutes at 94°C; 18 cycles at 94°C for 30 seconds, 58°C for 30 seconds, and 68°C for 30 seconds; final extension for 68°C for 5 minutes. Amplicons were tested for fragment size using a High Sensitivity DNA Kit (Agilent) on the Agilent 2100 Bioanalyzer. Each sample was quantified by KAPA Library Quantification Kit (KAPA Biosystems) on a StepOne Plus Real-Time PCR system (Applied Biosystems). The samples were then diluted to 28 pM and pooled into one library before emulsion PCR on the Ion Torrent One Touch 2 system (Life Technologies). The library was automatically enriched with the Ion OneTouch ES system. Once enriched, the library was loaded onto an Ion 314 Chip for high-throughput DNA sequencing on the Ion PGM next-generation sequencing platform, located in the Core Genomics Facility at the University of Hawaii at Hilo.

Drosophila sproati

To obtain DNA from microbes on the outside of the flies that were stored previously in 95% EtOH, flies were washed three times in sterile water. Each wash was centrifuged at 15,000g for 15 minutes, eluted with low TE (10mM Tris, 0.1mM EDTA pH8) and pooled to make the external microbe DNA sample. To remove any remaining microbe DNA from the outside the flies were then washed twice in 2.5% bleach and twice in sterile water. The final water wash was then spun down and eluted with low TE and tested with both bacterial and fungal primers to confirm sterility of the outside of the fly. To obtain a microbe DNA sample from inside of 5 male and 5 female flies using a Powersoil DNA isolation kit (MO BIO) according to manufacturer's instructions to extract DNA from the flies after washing.

Two primer sets were used to amplify V2-4-8 and V3-6,7-9 regions of the 16S rRNA gene as provided in the Ion 16S Metagenomics Kit (Life Technologies). This kit was chosen because multiple regions across the entire 16S rRNA gene are amplified. Previous studies have shown that these different regions provide differing levels of taxonomic resolution, which in turn allows us multiple options for downstream analysis (Barb et al. 2016). The PCR conditions were as follows: initial denaturation for 5 minutes at 95°C; 25 cycles at 95°C for 30 seconds, 58°C for 30 seconds, and 72°C for 20 seconds; final extension for 72°C for 7 minutes. To assess the fungal community, primers ITS1-F (5'-CTT GGT CAT TTA GAG GAA GTA A-3') and ITS2 (5'-GCT GCG TTC TTC ATC GAT GC-3') were used to amplify the internal transcribed spacer 1 region (Gardes and Bruns 1993). The PCR conditions were as follows: initial denaturation for 2 minutes at 95°C; 25 cycles at 95°C for 30 seconds, 56°C for 30 seconds, and 72°C for 30 seconds; final extension for 72°C for 5 minutes. Each sample had a specific barcode and Ion Torrent adapters ligated onto microbial amplicons using the Ion Plus Fragment Library Kit (Life Techonologies). Amplicons were tested for fragment size using a High Sensitivity DNA Kit (Agilent) on the Agilent 2100 Bioanalyzer. Each sample was quantified by KAPA Library Quantification Kit (KAPA Biosystems) on a StepOne Plus Real-Time PCR system (Applied Biosystems). Each sample was diluted to 50 pM and pooled into two libraries: 16S and ITS. Each library was loaded onto their own separate Ion 318v2 Chip BC for high-throughput DNA sequencing on the Ion PGM next-generation sequencing platform through the Core Genomics Facility at the University of Hawaii at Hilo

Bioinformatic Analysis

The Brazilian Microbiome Project's (BMP) 16S profiling analysis pipeline for Ion Torrent data was used to analyze bacterial 16S *D. sproati* metagenomic data (Pylro et al. 2014). The BMP pipeline combines tools from both QIIME and UPARSE methods of evaluating microbial communities. QIIME is an open source bioinformatics pipeline for used for microbial analysis (Caporaso et al. 2010). The QIIME pipeline was designed to analyze raw next-generation data across all major platforms (Illumina, 454, and Ion Torrent) and produce statistics and graphics at publication level quality. UPARSE utilizes an algorithm developed to construct highly accurate OTUs *de novo* from next-generation sequencing data. When comparing UPARSE and QIIME analyses on a mock community, the large majority of UPARSE OTUs were classified correctly in terms of reconstructions of biological sequences, as opposed to QIIME, where 23% to 67% of its OTUs were actually found to be chimeric sequences (Edgar 2013). The BMP pipeline utilizes UPARSE and QIIME based on their respective strengths to make an overarching bioinformatic pipeline that can analyze raw reads from the major next-generation sequencing platforms, accurately call OTUs, and produce microbial ecology related statistics and graphics.

Initial de-multiplexing, quality filtering, and de-replication was conducted utilizing USEARCH8 as a part of the UPARSE pipeline. UPARSE filtering is done based not on average Q score, but on the maximum expected error, which is a better indicator of read accuracy (Pylro et al. 2014). OTU clustering and chimera filtering was done using the UPARSE algorithm which performs both tasks simultaneously. This algorithm does not require technology or gene specific parameters such as an OTU size cutoff, algorithms, or data making it robust in terms of the data input (Edgar 2013). Once OTU clustering and filtering were finished, QIIME was used for the remainder of the data analysis. Taxonomy was assigned using the uclust method on QIIME. Sequences were aligned using greengenes reference sequences. Once sequences were aligned, they were filtered and used to make a reference tree and an OTU table. Taxonomy was then mapped onto the OTU table and diversity analysis was run in QIIME using the core_diversity_analyses.py script with the --sampling_depth parameter set to 726 (Appendix). The parameter "--sampling_depth" is the sequencing depth to use for even sub-sampling and maximum rarefaction depth. Sampling depth values were chosen based on minimum sequence depth of all analyzed samples. The BMP ITS profiling analysis pipeline for Illumina paired-end data was modified to analyze our fungal ITS *D. sproati* metagenomic data from the Ion Torrent (Appendix). Taxonomy was then mapped onto the OTU table and diversity analysis was run in QIIME using the core_diversity_analyses.py script with the "--sampling_depth" parameter set to 135.

Results

Culture-Based Yeast Survey

The yeast species that were cultured from *D. sproati* were as follows: *Candida cylindracea*, *Candida quercitrusa*, *Cyberlindnera lachancei*, *Prototheca ulmea*, and *Trichosporon porosum*. Yeast species on *D. ochracea* were *Candida boidinii*, *Candida cylindracea*, *Candida natalensis*, *Cyberlindnera lachancei*, *Phaffomyces sp. NF-2013*, *Phaffomyces opuntia*e, *Pichia sp. UWO(Stamps, Yang, Morales, & Boundy-Mills) 99-666.3*, *Pichia hawaiiensis*. Species identification were based off of BLAST sequence matches.

Yeast species cultured from rotting *Cheirodendron trigynum* were as follows: *Candida boidinii*, *Candida sp. ST-958*, *Cyberlindnera lachancei*, *Filobasidium uniguttulatum*, *Meyerozyma guilliermondii*, *Ogataea sp. 11-486*, *Rhodotorula mucilaginosa*, and *Rhodotorula slooffiae*. Yeast species cultured from rotting *Freycinetia arborea* were as follows: *Candida boidinii*, *Candida quercitrusa*, *Meyerozyma guilliermondii*, *Phaffomyces opuntiae*, and *Rhodotorula mucilaginosa*.

There was some overlap of yeast species between the Hawaiian *Drosophila* species and their respective host plant species. *Cyberlindnera lachancei* was cultured from both *D. sproati* and its host plant *Cheirodendron trigynum* (Figure 3). It is possible that *Cyberlindnera lachancei* may play a key role in this fly-host plant interaction. The same suggestion can be made for *Candida boidinii* and *Phaffomyces opuntiae* for *D. ochracea* and its host plant *Freycinetia arborea* as these are the only yeast species found on both the fly and its respective host plant (Figure 4).

Host Plant Metagenomics

Metagenomic analysis of the V6 region of the 16S rRNA gene revealed that *Freycinetia arborea*'s microbiota was found to be more diverse housing 20 genera within 11 bacterial families while *Cheirodendron trigynum* harbored 17 genera from 5 bacterial families (Figure 5). Enterobacteriaceae and Acidobacteriaceae were the dominate bacteria families found in both *C. trigynum* and *F. arborea*. *Cheirodendron trigynum*'s microbiota consisted of 67% Enterobacteriaceae while *Freycinetia arborea* was similarly composed at 64%. Enterobacteriaceae is a family of gram-negative bacteria that are common environmental bacteria that are associated with many plants and animals including *D. melanogaster* (Chandler et al. 2011, Madigan et al. 2010). The second most dominate bacteria family within each plant was Acidobacteriaceae. This bacteria family of primarily of acidophilic bacteria which are particularly abundant within soil (Eichorst et al. 2007). Acidobacteriaceae comprised 23% and 10% of the microbiota of *C. trigynum* and *F. arborea*, respectively.

Both host plants shared bacteria families Acidobacteriaceae, Enterobacteriaceae, Flavobacteriaceae, and Xanthomonadaceae while *Freycinetia arborea*'s microbiota also included bacterial families Burkholderiaceae, Comamonadaceae, Rhodobacteraceae, Shewanellaceae, Sphingobacteriaceae, Synechococcaceae, and Vibrionaceae. *Cheirodendron trigynum* and *Freycinetia arborea* share the genera *Citrobacter, Enterobacter, Erwinia, Granulicella, Klebsiella, Pantoea, Pectobacterium, Pseudoxanthomonas, Rahnella*, and *Terriglobus*.

There were differences at the genus level between each host plant. *Cheirodendron trigynum* microbiota included *Cronobacter, Edwardsiella, Ewingella, Raoultella, Salmonella, Weeksella,* and *Xanthomonas* while *Freycinetia arborea*'s microbiota included *Burkholderia,* *Flavobacterium, Pedobacter, Photobacterium, Roseobacter, Serratia, Shewanella, Synechococcus, Variovorax*, and *Yersinia*.

Drosophila Sproati Metagenomics

Bacteria Survey

The Ion Torrent sequencing run produced 3,719,939 DNA sequencing reads on the BC 318v2 BC chip with 49% loading (Figure 6). After loading, samples contained 100% enrichment, 77% clonal reads, and 87% of the final library remained after Ion Torrent PGM filtering. Samples averaged 155,173 reads, 31,354,860 bases with <Q20 score, and 219 mean read length (Table 1).

All *D. sproati* associated bacteria found in this study reside within 66 bacterial families. The most abundant families consist of Pasteurellaceae (50%), Halomonadaceae (12%), Porphyromonadaceae (8%), Pseudomonadaceae (4%), and Enterococcaceae (4%). Observed and expected (Chao1) bacteria OTU richness is higher in female flies than males. In Chao1 estimates that assume these groups are sampled exhaustively, the male fly associated bacterial community would consist of fewer than 25 OTUs, while female flies would have a maximum of 50 OTUs per any individual sample.

When separating fly samples by sex we found that the most abundant families of bacteria found in males were Pasteurellaceae (65%), Porphyromonadaceae (7%), Enterococcaceae (7%), Pseudomonadaceae (5%), and Acetobacteraceae (4%) while the most abundant female fly bacteria families consisted of Pasteurellaceae (33%), Halomonadaceae (24%), Porphyromonadaceae (10%), Sphingobacteriaceae (6%), and Xanthomonadaceae (4%) (Figure 13).

A weighted Unifrac Principle Coordinate Analysis (PCoA) revealed that when taking microbial composition and abundance into consideration, within *D. sproati* males, both bacterial communities found on the inside and outside of the flies group tightly together, while the internal and external microbe communities of female files did not. (Figure 14). This suggests that males may stay in one area while females may be more dispersed in their behavior or ecological relationships. When separating all *D. sproati* associated bacteria by location on the fly, the most abundant bacterial families inside the flies were Pasteurellaceae (56%), Porphyromonadaceae (11%), Enterococcaceae (6%), Pseudomonadaceae (6%), and Acetobacteraceae (5%), while the most abundant bacterial families found on the outside of the fly consisted of Pasteurellaceae (42%), Halomonadaceae (27%), Sphingobacteriaceae (6%), Porphyromonadaceae (5%), and Rhodobacteraceae (5%).

When assessing the internal microbiota of *D. sproati*, we found that 9 bacteria families reside within at least 90% of our samples. These families are Acetobacteraceae, Enterobacteriaceae, Enterococcaceae, Mycobacteriaceae, Neisseriaceae, Pasteurellaceae, Porphyromonadaceae, Pseudomonadaceae, and Xanthomonadaceae. This suggests that these bacteria families are likely to their make up the essential gut microbiota of *D. sproati* or are strongly associated with their diet.

Fungal Survey

The Ion Torrent sequencing run produced 7,352,309 DNA sequencing reads on the 318v2 BC chip with an 80% loading (Figure 8). After loading, samples contained 100% enrichment, 85% clonal reads, and 96% of the final library remained after Ion Torrent PGM filtering. Samples averaged 276,954 reads, 45,513,743 bases with a >Q20 score, and 184 base pairs in length (Table 2).

All *D. sproati* associated fungi found in this study reside within 10 fungal genera as well as 11 unknown fungal OTUs. The most abundant taxa include Cryptococcus (50%), Unknown Fungi 1 (15%), Rozellomycota (12%), Colletotrichum (5%), and Fellomyces (5%). Observed and expected (Chao1) fungal OTU richness is higher in male than female flies. In Chao1 estimates that assume these groups are sampled exhaustively, the fungal community associated with female flies are predicted to have fewer than 6 OTUs, while fungal community associated with male flies are predicted to have a maximum of 12 OTUs per any individual sample.

When analyzing fly samples by sex we found that the most abundant fungal genera found in male flies were Cryptococcus (37%), Unknown Fungi 3 (24%), Hannaella (14%), Unknown Fungi 1 (10%), and Bullera (9%), while the most abundant genera found in female flies consisted of Cyptococcus (52%), Unknown Fungi 1 (16%), Rozellomycota (14%), Colletotrichum (6%), and Fellomyces (6%).

A Bray-Curtis Principle Coordinate Analysis (PCoA) revealed that there are no clear relationships or grouping of samples between sex nor location of fungal microbiotia. When separating all *D. sproati* associated fungi by location on the fly, the most abundant fungal genera were Unknown Fungi 1 (5 0%), Colletotrichum (20%), Unknown Fungi 3 (11%), Cryptococcus (11%), and Bullera (4%), while the most abundant fungal genera found on the outside of the fly consisted of Cryptococcus (63%), Rozellomycota (17%), Fellomyces (7%), Candida (5%), and Unknown Fungi 1 (3%)

Discussion

Yeast Survey

The results of this study are consistent with previous research investigating the microbial communities associated with other *Drosophila* from around the world (Broderick and Lemaitre 2012, Chandler et al. 2011, Chandler et al. 2012). In this study we identified yeasts that are associated with *D. sproati* through both culture-based and culture-independent approaches, as well as yeasts associated with *D. ochracea* with a culture-based approach. The culture-based approach identified yeasts isolated on YM agar by sequencing the D1/D2 regions of the 26S rRNA gene, while culture-independent approach utilized next-generation high-throughput DNA sequencing approach to identify yeasts using the ITS1 region. For *D. sproati*, there was some overlap at the genus level, however the majority of diversity found using each method was unique. There was no major differences in the yeasts communities found associated with male or female flies either inside or outside the flies.

The genus *Candida* was found to be an abundant taxon using both methods, however the species of *Candida* isolated showed no overlap between the two methods. The culture-dependent method isolated the species *Candida cylindracea* and *Candida quercitrusa*, while high-throughput sequencing identified *Candida viswanathii* and *Candida sp. MM4035*. Additionally, high-throughput methods showed that the most abundant genus associated with *D. sproati* was *Cryptococcus*. Two species were identified, *Cryptococcus laurentii* and *Cryptococcus daszewskae* which together constitute approximately 50% of the sequenced reads. Previous studies have shown that species of *Cryptococcus* have been associated with other insects such as the leaf cutter ants *A. capiguara* and *A. laevigata*, as well as the moth *Sigelgaita sp*. (Pagnocca et al. 2008, Rosa et al. 1992). Members of *Cryptococcus* have also been found in the cactus

Pilosocereus arrabidae, an established host plant for *Drosophila serido* (Morais et al. 1994). *Cryptococcus laurentii* is among the most commonly isolated *Cryptococcus* species and has been heavily studied for its human pathogenic abilities (Kordossis et al. 1998) and antagonistic activity against post-harvest pathogens (Lima et al. 1998), however little is known about its relationship with insect hosts.

There was some overlap in the diversity of yeast species between the Hawaiian picture-wing *Drosophila* and their respective host plant species. *Cyberlindnera lachancei* was cultured from both *D. sproati* and its host plant *Cheirodendron trigynum* (Figure 3). Phaff et al. (1999), discovered *Cyberlindnera lachancei*, formerly named *Pichia lachancei* from rotting *Tetraplasandra*, *Clermontia,* and *Cheirodendron* on Hawaii Island. They postulated that this yeast species is vectored by one or several picture-winged *Drosophila*. By culturing it from *D. sproati*, we recovered evidence that supports this claim. The same can be postulated of *Candida boidinii* and *Phaffomyces opuntiae* for *D. ochracea* and its host plant *Freycinetia arborea,* as these are the only yeast species found on both the fly and its respective host plant (Figure 4). *Phaffomyces opuntiae*, formerly named *Pichia opuntiae* was originally discovered in decaying cacti in California and Australia, known host plants for cactophilic *Drosophila* (Starmer 1979). A Bray-Curtis dissimilarity Principle Coordinate Analysis (PCoA) of yeast communities found through our culture-independent approach found that there is no clear grouping of yeasts between samples separated by sex or location on *D. sproati*. It is possible that the majority of these yeasts are used purely for nutritional purposes. If that is the case, the diversity of yeast species the *Drosophila* consume is not as important, as long as specific nutritional requirements are met.

Culturing identified five unique taxa, while 26 OTUs were found via high-throughput sequencing. There are several possible explanations for this difference. It is estimated that most fungi are likely non-cultivable (CITE). Culturing techniques are limited by the nutrients supplied to the microbe, so it is likely that the taxa found through the culture-independent approach were not supplied their essential nutrients in the YM agar used. It is also possible that due to the 26S region being a much more conserved region than ITS1, there might not have been enough resolution to distinguish between very closely related taxa. Lastly, the difference could be explained by the sampling differential. The culture-dependent approach was done on a single female *D. sproati* while the culture-independent approach was conducted on five male and five female *D. sproati.* Despite these limitations, the lack of overlap in diversity between culture-based and high-throughput approaches reveals them as complementary, which has been suggested by a variety of other studies (reviewed in Handelsman 2004).

Bacteria Survey

The bacterial communities associated with the *Drosophila* host plants, when utilizing the culture-independent approach, showed overlap between *D. sproati* and its host plant *C. trigynum*. Enterobacteriaceae dominated the bacterial microbiota of *C. trigynum* and was among the 10 most abundant families found associated with *D. sproati*. In a global survey of bacterial microbiomes of *Drosophila*, it was found that Enterobacteriaceae was present in almost all of the 14 wild caught species, as well as laboratory stock populations (Chandler et al 2011). Within Enterobacteriaceae there are individual species which have been shown to be essential for nutrition in aphids, as well as aid in defense from parasites (Douglas 1998, Oliver et al. 2003). It is possible that some of the species within Enterobacteriaceae form a symbiotic relationship with

D. sproati, however with our approach it is not possible to confirm which bacteria are residential and which are transient. Since many of the families of bacteria were found on both *D. sproati* and its host plant, it also possible that the flies can vector these microbes to rotting plant material.

There was a difference in the bacterial microbiome diversity of the *Drosophila* host plants within this study. *Freycinetia arborea*'s microbiota was more diverse with 20 genera within 11 bacterial families. In contrast, *C. trigynum* had a lower diversity with 17 genera from 5 bacterial families (Figure 5). Enterobacteriaceae and Acidobacteriaceae were the dominate bacteria families found in both *C. trigynum* and *F. arborea*. *Cheirodendron trigynum*'s microbiota consisted of 67% Enterobacteriaceae while *Freycinetia arborea* was similarly composed at 64%. Acidobacteriaceae comprised 23% and 10% of the microbiota of *C. trigynum* and *F. arborea* respectively. These results suggest that these two dominant bacterial families may be key microbial members of suitable Hawaiian picture-wing *Drosophila* host plants. While there are findings linking Enterobacteriaceae to *Drosophila*, both this study and the results from Chandler et al (2011) did not sequence any OTU's belonging to bacteria within Acidobacteriaceae.. Although Acidobacteriaceae is a dominant family present on the host plants, its presence does not necessarily constitute a relationship with the insects that utilize the plant as an ovipositional site. One possible explanation for the high abundance of Acidobacteriaceae found in this study is that our samples were taken from rotting material of these plant species. When living plant tissue is wounded, yeasts and fungi begin to infect the plant, facilitating breakdown of plant tissue and causing rot. Fungi release organic acids which can lower external pH. Some aggressive tissue-rotting pathogens, such as *Sclerotinia sclerotiorum* in plants, are found to release large amounts of oxalic acid, which can lower the pH as low as 4.0 (Noyes and

Hancock 1981). This can create a suitable environment for acidophilic bacteria such as those found within Acidobacteriaceae.

High-throughput sequencing results of the bacterial community associated with *D. sproati* were similar to previous studies with 16 out of 17 of the most abundant bacterial orders found by Chandler et al. (2011) were found within *D. sproati*. Chandler et al. (2011) also found that four bacterial families representing three orders make up 90% of all sequences within their *Drosophila*. These bacteria were Enterobacteriales: Enterobacteriaceae (60%), Rhodospirillales: Acetobacteraceae (9%), and Lactobacillales: primarily Lactobacillaceae and Enterococcaceae (21%). The bacteria found on the inside of *D. sproati* also showed the same three families Acetobacteraceae, Enterobacteriaceae, and Enterococcaceae among the most abundant families. However, Lactobacillaceae was not present in *D. sproati* (Figure 14). Interestingly, we also found that the family Pasteurellaceae dominated *D. sproati* males, females, internal, and external microbiomes. This order constitutes 50% of all sequences found within our dataset. Pasteurellaceae is made up of at least 70 species in 15 genera. They range from commensal to opportunistically pathogenic in various birds and mammals, including humans. (Christensen and Bisgaard 2008). Species of Pasteurellaceae have also been found associated with insects such as an aphid, a ground beetle, honeybee, and *Drosophila* (Chandler et al 2011). Little is known about the possible role Pasteurellaceae has within *Drosophila*, nor any other insect. Further investigation is needed to determine whether there is a mutualistic relationship taking place or a purely commensal role between the bacteria and fly. One possible explanation for this discrepancy are the ecological differences between the *Drosophila* sequenced by Chandler et al. (2011) and the picture-wing species investigated here. Only *Drosophila* that were associated with fruit, flowers, mushrooms, or cacti were investigated by Chandler et al. (2011), as opposed to *D. sproati,* in this study, which is a bark breeder. The substrate on which *Drosophila* feed or breed is likely to have an effect on both their internal and external microbiome (Chandler et al. 2011, Staubach et al. 2013, Sharon et al. 2010).

We also compared the external microbiota of naturally occurring *D. sproati* with the internal microbiota. A weighted Unifrac Principle Coordinate Analysis (PCoA), which takes into account both composition and abundance, showed that the internal and external microbiomes grouped tightly in males, but not in females. This suggests that males may stay in one area while females may be more dispersed in their behavior or ecological relationships. Lekking behavior may be a possible explanation for the tight grouping of male microbiomes. A lek is an aggregation of males that gather to engage in competitive displays that may entice visiting females who are surveying prospective partners for copulation. Hawaiian picture-wing *Drosophila* are known to perform this behavior while other continental species do not. This was also evident when raising *D. sproati* in a laboratory setting for this study, we found that there must be a minimum of 3 males and 3 females placed in one jar in order for mating and copulation to take place. It is possible that while males are all grouped together, they are staying in one restricted area and mostly feeding on the same material, and therefore gaining very similar bacterial microbiomes. In contrast females may be flying around looking for potential mates at these lekking sites that are not necessarily tied to the host plant of these flies and then also searching for appropriate host plant for oviposition.

A recent study comparing the internal and external bacterial microbiomes of *D. melanogaster* lab stocks found that the intestinal bacterial microbiome represents a highly reduced subset of the external bacterial communities (Chandler et al. 2011). The findings here indicate that some bacteria families were present internally and not externally, and the relative amounts of internal bacterial families could be similar to that of the external families found on the fly (Figure 13). These differences indicate that the natural external and internal microbiota acquired by flies may be more diverse in nature than those procured by flies in controlled laboratory settings. In laboratory settings, variables such as food and suitable oviposition substrate are less variable, which may be important in determining if altering environmental factors can affect diversity of the microbiome and even fly survivorship. However, due to the generalist nature of *D. melanogaster*, microbial studies on specialist species, such as the picture-wing *Drosophila* studied here, may provide key insights into how symbionts are horizontally transferred through the environment, as well as the nature of host-preference.

Microbial surveys provide insight into the interplay between insect, plant host, and microbe, but due to the limitations of these studies, we cannot differentiate between transient or resident microbes, and specifically when utilizing a culture-independent approach, we cannot distinguish between living or dead bacteria found within our samples. However, when assessing the internal microbiota of *D. sproati*, we found that 9 bacterial families reside within at least 90% of our samples. These families are Acetobacteraceae, Enterobacteriaceae, Enterococcaceae, Mycobacteriaceae, Neisseriaceae, Pasteurellaceae, Porphyromonadaceae, Pseudomonadaceae, and Xanthomonadaceae. This suggests that these bacterial families are more likely to make up the essential gut microbiota of *D. sproati* or are strongly associated with their diet.

Chapter 3
The Effect of Microbes on *Drosophila sproati* Mortality and Epicuticular Hydrocarbons

Introduction

***Drosophila*, Microbes and Cuticular Hydrocarbons**

Drosophila species, and most insects, contain a layer of lipids, hydrocarbons and other compounds on the surface of their cuticles (Coyne 1996). These epicuticular hydrocarbons (CHCs) are typically chemically simple molecules which can be placed in three major groups; alkanes, alkenes, and methyl branched hydrocarbons. These molecules are produced in a specialized cells called oenocytes and are then transported to the surface of the cuticle (Diehl 1975). These insect CHCs serve a dual function in insects, aiding in both desiccation prevention and chemical signaling (Chung and Carroll 2015).

Maintaining water levels is critical for insect survival (Edney 1967; Howard & Blomquist 1982). The water-proof layer of CHCs range in length from 21 to 50 carbons long and provide the primary control of water loss in insects (Gibbs and Pomonis 1995). The removal of these CHCs through either physical or genetic manipulation results in a high sensitivity to desiccation (Chung and Carroll 2015). In general, as the length of the CHC increases, melting point increases. However, composition of the CHC also determines melting point, where unsaturated and methyl-branched hydrocarbons generally have lower melting temperatures (Gibbs and Pomonis 1995). *Drosophila mojavensis*, a cactophilic fly found in the deserts of southwest USA and Mexico have evolved longer chain CHCs than their more temperate relatives to further protect themselves from the higher stress of desiccation that deserts provide (Etges and Ahrens 2001).

Chemical communication in insects are primarily used for mate recognition but can also affect other behaviors such as learning, aggregation, and dominance (Blomquist and Bagneres

2010). Insect pheromones used in chemical communication are generally divided into two groups; primer and releaser compounds (Wilson 1963). Epicuticular hydrocarbons are a part of the releaser class of pheromones which initiate an immediate behavioral response upon reception. Due to these hydrocarbons forming such long chains, they become waxes and are used as contact pheromones involved in species and sex discrimination in Dipterans (Alves et al. 2010).

Microbial communities associated with *Drosophila* have been implicated in the expression of epicuticular hydrocarbons. Fedina et al. (2012) determined that a high yeast diet significantly increased the total concentrations of various CHCs in *D. melanogaster*. However, whether these specific CHCs have defined pheromonal functions or require dietary yeast to be synthesized is still unknown. Sharon et al. (2010) showed that the bacterium *L. plantarum* can alter these cuticular hydrocarbon sex pheromones, suggesting that these symbiotic bacteria can influence mating preference through altering pheromone levels.

Methods

Hawaiian *Drosophila* Population Rearing

Drosophila sproati were maintained in a controlled-environmental room, following specific Hawaiian *Drosophila* rearing protocol using host-plant material and food media. Rearing room conditions remain at a constant 18°C, 70% RH with a 12:12 light: dark cycle. Adults were housed in 1-gallon breeding jars containing adult-food, yeastless wheeler-clayton medium with a small piece of tissue moistened with a tea made of *Cheirodendron trigynum*. The moistened tissue maintains optimal humidity and the host plant tea acted as an ovipositional site while the wheeler-clayton medium serve as a food source (Wheeler and Clayton 1965). Adult food vials were replaced every four to five days for the duration of the study.

Mortality Study

For the mortality study, *D. sproati* individuals were captured in the wild, and raised in the laboratory. The starting population for laboratory stock lines consisted of ~40 individuals. The second in-laboratory generation was then split into four treatment groups: control, antibacterial treatment group, antifungal treatment group, and a group with both antibacterial and antifungal treatments. In the control group the tea and food was prepared according to the standard Hawaiian *Drosophila* protocol (see above). In the antibacterial group the tea and food was identical to the control group with the addition of 100 ug/ml of chloramphenicol. Similarly, in the antifungal group the tea and food was identical to the control group with the addition of 100 ug/ml of cycloheximide (Sigma). Lastly, in the group with both antifungal and antibacterial treatment the tea and food was supplemented with both 100µg/ml chloramphenicol and 100µg/ml cycloheximide.

This study was conducted in two blocks. Each block contained 2-3 replicates per treatment. Replicates in each treatment group contained 3 males and 3 females housed together in a 1-gallon breeding jar. Jars were checked every 1-2 days for fly survivability. Once a dead fly was found, it was immediately removed from the 1-gallon breeding jar and sexed.

R 3.2.3 statistical program was used to determine the effect of antibacterial and antifungal effect on lab stock *D. sproati* mortality. A Cox proportional hazard model and two-way nested ANOVA was used to determine the effect of treatment, sex, and block(jar) on mortality. A post hoc Tukey's multiple comparison test was used for all significant relationships found in the two-way nested ANOVA.

Epicuticular Hydrocarbon Extraction and Gas Chromatography

To investigate the effect of presence or absence of bacterial communities on *Drosophila* epicuticular hydrocarbons lab stock *D. sproati* populations, that were the second generation from the wild-caught flies, were split into two groups: control and antibiotic. In the control group the tea and food was prepared in standard Hawaiian *Drosophila* protocol (see above). In the antibacterial group with the tea and food that was identical to the control group except for the addition of 100µg/ml chloramphenicol. The treatments occurred over two generations with one generation adults exposed to the treatments and again a second generation that was exposed to the treatments throughout entire life cycle and into adult stage These second generation exposed adult flies were removed from treatment and control vials three weeks after eclosion and prepared for hydrocarbon extractions. This time period was chosen because a prior study found that other Hawaiian *Drosophila* within the picture wing clade, take 2-3 weeks to mature depending on sex (Craddock and Boake 1992).

To extract the epicuticular hydrocarbons, each fly was placed in a 4ml vial and held at -80°C for ten minutes for euthanization. To each vial containing a fly, 1ml of hexane was added and gently shaken for 10 minutes. The solvent was pippeted out of the vial and transferred to a new clean 2ml vial and nitrogen gas was used to evaporate excess hexane. Extracts were stored at -80°C until used for analysis.

A gas chromatograph system equipped with a 30 meter Agilent technologies HP-5MS high resolution gas chromatography column and a mass selective detector was used in this study. The GC was preheated to 180°C prior to injection of the sample. After injection the sample was held at 180°C for one minute, followed by a 3°C increase per minute for 46 minutes, ending the cycle at 320°C. After each sample had been analyzed using the GC, amounts of each major hydrocarbon in each fly was quantified. Major CHCs of individuals within each experimental group was averaged to determine the percentage value for each major CHC in the profile.

R 3.2.3 statistical program was used to determine the significant differences that existed in CHC production between control lab stock *D. sproati* and *D. sproati* that has undergone antibacterial treatment. An initial Shaprio-Wilk test for normality was conducted on each individual fly's epicuticular hydrocarbons. A two-factor ANOVA, post hoc Tukey's multiple comparison test, and Principle Component Analysis (PCA) were conducted on the effect of sex and treatment on each individual CHC. The labdsv statistical package was used to conduct PCA in R 3.2.3. A follow up two-factor ANOVA was conducted on the effect of sex and treatment on each principle Component.

Results

Mortality Study

A Cox proportional hazard model showed that treatment had a significant effect on mortality (z=6.44, df=3, p=1.2e-10), while sex did not (Table 5). A two-way nested ANOVA showed that treatment had a significant effect on mortality (F=553.9, df=3, p=2.20e-16), while sex did not (F=0.5236, df=1, p=0.47118) (Table 7). A post hoc Tukey's multiple comparison revealed that control/antibacterial and both/antifungal interactions did not significantly differ, while antibacterial/antifungal, both/antibacterial, control/antifungal, and control/both interactions were significantly different (Table 6).

Throughout this 40-day study, flies that were exposed to treatments that contain antifungal survived a maximum of 18 days averaging at 10.6 days, and resulted in 0 flies surviving till the end of this 40-day study. Alternatively, flies who were exposed to the control and antibacterial treatment's survivability were drastically higher at 37.5 days and 39.2 days respectively, with a combined 90.7% survival till the end of this 40-day study (Figure 22). This data suggests that *D. sproati* need fungi to survive but apparently can live without much of the bacteria normally present.

Gas Chromatography

The major CHCs for *D. sproati* were 11,13-methylpentacosane (11+13MeC25), 2-methylhexacosane (2MeC26), 11,13 methylheptacosane (11+13Me C27), 2-methyloctacosane (2MeC28), as well as 5 unknown CHCs (Table 8). A two-factor ANOVA and post hoc Tukey's multiple comparison revealed several significant differences between sex and treatment (Table). Males within the antibacterial treatment showed a significant difference in the production of

Unknown 1 and 2MeC28 (Table 19, 26). Unknown 1 was 191% greater in antibacterial treated males compared to control males, going from 6.92% of the control male's percent CHC composition to 13.19% of the antibacterial male's percent CHC composition. In contrast to Unknown 1, 2MeC28 showed a decrease of similar magnitude in antibacterial treated male flies. For example, 2MeC28 made up 13.87% of control male's percent CHC composition and decreased to 6.36% in the antibacterial treated male flies. Female fly's CHC profile did not significantly differ between treatments.

There was a significant interaction between male and female fly epicuticular hydrocarbons across the treatments: Unknown 1 ($F = 14.18$, $df = 1$, $p = 0.0021$), 11,13MeC27 ($F=5.925$, $df = 1$, $p = 0.2891$), and 2MeC28 ($F = 13.85$, $df = 1$, $p = 0.00228$). Male and female flies within the control group significantly differed in Unknown 1, Unknown 3, Unknown 4, Unknown 5, and 2MeC28 percentages (Table 19, 21, 22, 23, 26), while males and females within the antibacterial treatment significantly differed in 11+13MeC25, Unknown 3, Unknown 4, Unknown 5, 11+13MeC27 (Table 18, 21, 22, 23, 25). This shows that when *D. sproati* had undergone antibacterial treatment, sexual dimorphism in their respective CHCs shift.

The principle component analysis resulted in PC1 explaining 64.2% with a large negative loading from females and PC2 explaining 17.2% of the variation (Table 27). A two-factor ANOVA revealed that sex was significantly different for PC1 ($F = 49.24$, $df = 1$, $p = 6.08e-06$) (Table 28) and treatment was significantly different for PC2 ($F = 21.05$, $df = 1$, $p=0.0004$) (Table 29).

Discussion

Mortality Study

The relationship between *Drosophila* and yeast has been extensively studied (Baumberger 1917, Heed et al. 1976, Anagnostou et al. 2010a, Stamps et al. 2012), however with new sequencing technologies many recent studies have opened new lines of investigation into the importance of fungi to *Drosophila*. The majority of *Drosophila* are saprophages, feeding on dead or decaying organic matter (Magnacca et al. 2008). Yeasts are often necessary to convert decaying matter into the dietary factors on which *Drosophila* depend. For example, axenic flies, i.e. germ-free, were unable to survive on sterile banana, a carbohydrate-rich substrate, without the addition of dietary yeasts (Anagnostou et al. 2010a). Bacteria have also been found to be influential for *Drosophila* fitness. A recent study found that the presence of bacteria during the first week of adult life can enhance lifespan, despite unchanged food intake. The same study also found that the presence of bacteria later in life can reduce *Drosophila* life span, demonstrating that there is a dynamic interaction between bacteria and longevity (Brummel 2004).

The results of this study demonstrate that the presence or absence of the associated microbiota have a dramatic effect on the survivability of *D. sproati*. The antifungal and the combined antifungal and antibacterial treatment groups had a significant negative effect on adult survivorship, while there was no significant difference between males and females, from the Cox proportional hazard model (Table 5). Throughout the 40-day study, flies that were exposed to treatments that contained antifungal agents survived a maximum of 18 days, with an average of 10.6 days, and resulted in no flies surviving to the termination of the study (40 days). This suggests that *D. sproati* relies on the fungi associated with the host plant tea or that the antifungals detrimentally affected their internal fungal microbiota. These results are consistent with a large body of literature showing the importance of yeasts as an essential food source for most *Drosophila*. An alternate explanation for the high mortality within the antifungal containing treatments could be the negative effects caused by cycloheximide, the antifungal chemical used for treatment of the host plant tea and adult fly food. This is the first study to investigate the effect of cycloheximide on Hawaiian *Drosophila* and more research is needed to separate the effects of the lack of fungi and the fungicide on *D. sproati.*

Interestingly, flies exposed to the control and antibacterial treatments had high survivorship with a mean of 37.5 days and 39.2 days, respectively, and a combined 90.7% survival until the end of this 40-day study (Figure 22). These results indicate that bacteria associated with the host plant and adult food created in the laboratory are not obligatory to any stage of *D. sproati*'s life cycle. However, previous studies have found that through the use of axenic *Drosophila melanogaster* flies, the lack of an expansive bacterial microbiome caused a significantly delayed development, primarily caused by growth defects during the larval stage. Studies have also shown that eye color can be affected by associated bacteria, which suggests that the microbiota plays a role in the optimization of host metabolism (Broderick and Lemaitre 2012). This suggests that even though bacteria may not be necessary for survival; the fitness of *Drosophila* is likely affected by its associated microbiota.

While the respective microbial communities in host plant tea and food were likely substantially reduced in the antibiotic and antifungal treatments in this study, it is not certain if the flies themselves were sterilized. Further investigation would be required to assess if the antibiotic or antifungal treatments used in this study were able to act on the fly's internal microbiome. A possible test would be conducting a metagenomics survey of microbiomes associated with flies under treatment to check for the presence of bacteria or fungi. Additionally, antibiotic studies of fly survivorship should be conducted over multiple generations in order to further assess if the reduction of the bacterial microbiota negatively affects population-level fitness.

Epicuticular Hydrocarbon Analysis

Although hydrocarbons are chemically simple, insects have evolved to utilize them for a wide range of purposes such as desiccation prevention, a barrier to microorganisms, as well as pheromonal communication (Howard and Blomquist 2004). In *Drosophila,* long-chain epicuticular hydrocarbons form waxes and are often used in chemical communication as contact pheromones (Ferveur et al 1997). While there has been research into both the genetic and many non-genetic factors such as temperature, food, and development in terms of CHC production in *Drosophila*, the role of the *Drosophila* microbiome and associated microbes remains largely unknown.

We found that *D. sproati*'s major CHCs consist of 11+13-methylpentacosane (11+13MeC25), 2-methylhexacosane (2MeC26), 11+13-methylheptacosane (11,13MeC27), 2-methyloctacosane (2MeC28), as well as 5 unknown CHCs. Under control conditions, Unknown 1, Unknown 3, Unknown 4, Unknown 5, and 2MeC28 were sexually dimorphic. These results are consistent with previous CHC analyses which showed many instances of sexually dimorphic CHCs across 27 Hawaiian *Drosophila* (Alves et al. 2010, Fezza 2014). The differences in CHCs between males and females may indicate that these compounds could play a communication role to aid in distinguishing sex within the species or possibly mate choice.

When exposed to the antibiotic treatment, males exhibited a significant difference in the production of Unknown 1 and 2MeC28 (Table 19, 26). Unknown 1 was 191% greater in antibacterial treated males compared to control males, going from 6.92% of the control male's percent CHC composition to 13.19% of the antibacterial male's percent CHC composition. In contrast to Unknown 1, 2MeC28 showed a decrease of similar magnitude in antibacterial treated male flies. For example, 2MeC28 made up 13.87% of control male's percent CHC composition and decreased to 6.36% in the antibacterial treated male flies. These results show that the lack of bacteria on either the host plant tea, adult food, or fly itself impact the production of CHCs of *D. sproati*. The cause of this relationship has yet to be elucidated. One possible explanation is that the lack of bacteria causes a nutritional deficit that makes flies allocate resources differently or away from the regulation of these CHCs. Another explanation is that the microbes themselves are physically acting on these CHCs, altering the CHC production levels. Microorganisms within the gut tract of the termite *Zootermopsis nevadensis* were found to supply a necessary hydrocarbon precursor to its epidermal cells for use in hydrocarbon biosysnthesis (Guo et al. 1991). It is also possible that a similar relationship occurs between *D. sproati* and its microbiota, which would explain our results. Surprisingly, female fly CHCs did not significantly differ between treatments. Due to both Unknown 1 and 2MeC28 being sexually dimorphic CHCs found on *D. sproati*, it is likely that these CHCs act as a sex pheromone, however more evidence would be needed to confirm this hypothesis. Behavioral assays would need to be conducted to further elucidate the role of these to particular CHCs.

The epicuticular hydrocarbon 2-methyloctacosane (2MeC28) has been known to function as a contact pheromone. In the hardwood stump borer beetle *M. dasystomus*, 2MeC28 along with 2MeC26 were found to make up 17% of their CHC profile. Bioassays were conducted to further understand the role of these two hydrocarbons. A previous study found that when evaluating a mating trial between a dead female coated with either 2MeC26 or 2MeC28, males did not regularly display mating behavior. However, when these two compounds were both utilized in the same mating trial, full progression of mating behavior was reinstated (Spikes et al. 2010). These results show that both 2MeC26 and 2MeC28 act as contact pheromones, and suggest that a combination of these CHCs are needed to elicit a response. In Hawaiian picture-wing *Drosophila*, 2MeC28 has been shown to have an effect on mating, where an increased amount of 2MeC28 correlated with a decreased copulation success between backcrossed *Drosophila silvestris* males and *Drosophila silvestris* females (Fezza 2014).

Sharon et al. (2010) showed that the microbiota of *Drosophila* can influence mating behavior. After rearing an isogenic population of flies on either starch or molasses media, flies exhibited a significant mating preference for partners reared on the same medium. The switch in diet led to changes in gut microbiota composition and an antibacterial treatment abolished mating preference, suggesting that the fly microbiota is important in mate detection or success. Furthermore, antibacterial-treated flies re-associated with a mix of *Lactobacillus spp*. isolated from molasses-reared flies or mono-associated with a *Lactobacillus plantarum* isolated from starch-reared flies exhibited mating preference. In comparing the cuticular hydrocarbons, differences were observed between the starch and molasses-reared flies, suggesting that associated microbiota were influencing mating preference by altering sex pheromones. This study points out how microbiota, through an impact on mating preference, may contribute to speciation and evolution. As fly populations living on different diets may be geographically separated to some extent, diet induced mating preference could further reduce interbreeding of the populations, reinforcing species barriers, and possibly leading to speciation.

Tables

Table 1. Ion Torrent 16s Bacteria Sequencing Information

Sex	Location	Sample Number	Bases	>Q20	Reads	Mean Read Length
Male	Inside	1	52,017,629	47,825,876	239,747	217 bp
Male	Inside	2	37,366,284	34,469,321	166,086	225 bp
Male	Inside	3	42,447,427	39,070,638	198,159	214 bp
Male	Inside	4	40,422,969	37,485,867	174,187	232 bp
Male	Inside	5	43,050,442	39,387,307	204,961	210 bp
Female	Inside	1	33,061,763	30,064,733	165,317	200 bp
Female	Inside	2	33,061,419	30,237,299	175,349	189 bp
Female	Inside	3	30,123,473	27,356,938	145,528	207 bp
Female	Inside	4	36,430,893	33,647,821	174,545	209 bp
Female	Inside	5	33,490,888	30,937,446	154,750	216 bp
Male	Outside	1	10,983,098	10,161,801	49,377	222 bp
Male	Outside	2	40,288,103	37,520,992	179,099	225 bp
Male	Outside	3	35,912,852	33,349,773	156,714	229 bp
Male	Outside	4	36,248,832	33,513,497	166,422	218 bp
Male	Outside	5	32,318,639	29,722,128	149,398	216 bp
Female	Outside	1	40,613,544	37,244,420	169,522	240 bp
Female	Outside	2	39,522,742	35,803,862	156,152	253 bp
Female	Outside	3	32,625,952	30,116,639	147,745	221 bp
Female	Outside	4	33,215,701	30,795,152	139,806	238 bp
Female	Outside	5	34,067,169	31,549,380	153,810	221 bp
Averages			34,064,076	31,354,860	155,173	215 bp

Table 2. Ion Torrent ITS1 Bacteria Sequencing Information

Sex	Location	Sample Number	Bases	>Q20	Reads	Mean Read Length
Male	Inside	1	30,528,121	25,056,837	197,186	155 bp
Male	Inside	2	60,712,872	50,937,190	291,450	208 bp
Male	Inside	3	60,830,507	54,360,457	286,578	212 bp
Male	Inside	4	62,135,450	54,659,546	279,908	222 bp
Male	Inside	5	57,371,852	49,577,761	303,437	189 bp
Female	Inside	1	28,496,415	23,811,707	187,924	152 bp
Female	Inside	2	34,385,150	29,139,468	211,942	162 bp
Female	Inside	3	50,495,546	43,836,774	273,596	185 bp
Female	Inside	4	48,861,148	40,541,902	297,027	165 bp
Female	Inside	5	56,964,221	51,169,312	255,978	223 bp
Male	Outside	1	37,752,762	31,355,788	231,591	163 bp
Male	Outside	2	53,813,071	46,876,351	268,525	200 bp
Male	Outside	3	159,224,127	141,202,875	740,882	215 bp
Male	Outside	4	71,482,430	62,640,619	372,517	192 bp
Male	Outside	5	68,761,163	58,591,152	452,727	152 bp
Female	Outside	1	74,822,278	65,816,110	398,134	188 bp
Female	Outside	2	51,223,504	44,424,038	301,299	170 bp
Female	Outside	3	72,326,165	61,480,343	374,271	193 bp
Female	Outside	4	36,267,329	33,203,554	158,200	229 bp
Female	Outside	5	51,510,607	44,729,491	265,314	194 bp

Table 3. Comparison of α-diversity measurements between bacterial sampling of *Drosophila sproati.*

	Observed OTUs Average	Observed OTUs Error	Chao1 Average	Chao1 Error
Male Inside	14.34	4.089	17.995	5.463
Male Outside	19.12	10.101	24.188	12.046
Female Inside	38.38	28.477	49.527	37.491
Female Outside	27.42	12.306	41.483	17.845

Table 4. Comparison of α-diversity measurements between fungal sampling of *Drosophila sproati*.

	Observed OTUs Average	Observed OTUs Error	Chao1 Average	Chao1 Error
Male Inside	4	1.824	4.55	2.432
Male Outside	9.325	3.439	11.658	4.82
Female Inside	3.433	1.096	3.55	1.188
Female Outside	4.66	2.359	5.315	2.926

Table 5. Cox proportional hazard model testing the factors and interactions of sex and treatment on mortality. Likelihood ratio test = 92.9 on 3 df,. p = 0, n=114, and the number of events = 65. Significant p-values are bolded.

	coef	exp(coef)	se(coef)	z	p
Sex	1.579	4.852	0.947	1.67	0.095
Treatment	1.492	4.447	0.232	6.44	**1.20E-10**
Sex:Treatment	-0.48	0.619	0.269	-1.78	0.075

Table 6. Tukey’s pairwise comparisons the factors and interaction of treatment on mortality. Significant p-values are bolded.

	diff	lwr	upr	p adj
Antifungal-Antibiotic	-29.475	-32.0727	-26.8773	**0**
Both-Antibiotic	-27.7417	-30.3393	-25.144	**0**
Control-Antibiotic	-1.70833	-4.30598	0.889317	0.318774
Both-Antifungal	1.733333	-0.71576	4.182422	0.256026
Control-Antifungal	27.76667	25.31758	30.21576	**0**
Control-Both	26.03333	23.58424	28.48242	**0**

Table 7. Two-way Nested ANOVA testing the factors and interactions of sex, treatment, and block(jar). Significant p-values are bolded.

	Df	Sum Sq	Mean Sq	F Value	Pr(>F)
Sex	1	6.9	6.9	0.5236	0.47118
Treatment	3	21827.1	7275.7	553.8977	**2.20E-16**
Block	1	64.2	64.2	4.8889	**0.02954**
Block:Jar	14	864.2	61.7	4.6996	**2.24E-06**
Sex:Treatment	3	80.1	26.7	2.0335	0.11473
Residuals	91	1195.3	13.1		

Table 8. Main CHCs of *Drosophila sproati* flies between each treatment. Averages are bolded.

	Control Males		Antibiotic Males		Control Females		Antibiotic Females	
	Average	**SD**	**Average**	**SD**	**Average**	**SD**	**Average**	**SD**
11+13Me C25	**21.61%**	3.17%	**21.81%**	3.56%	**28.42%**	3.59%	**34.96%**	3.15%
Unknown 1	**6.92%**	0.86%	**13.19%**	2.48%	**16.17%**	3.33%	**13.94%**	1.95%
Unknown 2	**6.19%**	1.61%	**6.32%**	1.04%	**7.14%**	0.75%	**5.23%**	0.65%
Unknown 3	**9.96%**	1.09%	**10.56%**	0.46%	**7.81%**	0.79%	**7.34%**	1.08%
Unknown 4	**16.95%**	2.30%	**20.02%**	2.38%	**11.61%**	3.47%	**11.55%**	1.59%
Unknown 5	**7.69%**	0.69%	**7.14%**	1.78%	**3.49%**	1.49%	**3.27%**	0.92%
2Me C26	**12.73%**	2.13%	**11.27%**	3.05%	**12.82%**	2.14%	**8.75%**	2.95%
11+13Me C27	**4.07%**	0.91%	**3.34%**	0.70%	**4.79%**	0.82%	**7.22%**	2.51%
2Me C28	**13.87%**	3.60%	**6.36%**	1.61%	**7.74%**	1.06%	**7.74%**	1.52%

Table 9. Two-way ANOVA results testing the factors and interaction of sex and treatment on 11,13MeC25. Significant p-values are bolded.

	Df	Sum Sq	Mean Sq	F Value	Pr(>F)
Sex	1	440.54	440.54	38.1375	**2.41E-05**
Treatment	1	41.76	41.76	3.6156	0.07802
Sex:Treatment	1	43.75	43.75	3.7871	0.07201
Residuals	14	161.72	11.55		

Table 10. Two-way ANOVA results testing the factors and interaction of sex and treatment on Unknown 1. Significant p-values are bolded.

	Df	Sum Sq	Mean Sq	F Value	Pr(>F)
Sex	1	84.749	84.749	15.274	**0.001576**
Treatment	1	25.551	25.551	4.605	**0.049895**
Sex:Treatment	1	78.679	78.679	14.18	**0.002088**
Residuals	14	77.679	5.549		

Table 11. Two-way ANOVA results testing the factors and interaction of sex and treatment on Unknown 2. Significant p-values are bolded.

	Df		Sum Sq	Mean Sq	F Value
Sex	1	0.0302	0.0302	0.0262	Pr(>F)
Treatment	1	2.832	2.832	2.449	0.87382
Sex:Treatment	1	4.5399	4.5399	3.926	0.13992
Residuals	14	16.1895	1.1564		0.06754

Table 12. Two-way ANOVA results testing the factors and interaction of sex and treatment on Unknown 3. Significant p-values are bolded.

	Df	Sum Sq	Mean Sq	F Value	Pr(>F)
Sex	1	33.379	33.379	46.5156	**8.32E-06**
Treatment	1	0.057	0.057	0.0798	0.7817
Sex:Treatment	1	1.251	1.251	1.7432	0.2079
Residuals	14	10.046	0.718		

Table 13. Two-way ANOVA results testing the factors and interaction of sex and treatment on Unknown 4. Significant p-values are bolded.

	Df	Sum Sq	Mean Sq	F Value	Pr(>F)
Sex	1	231.008	231.008	36.6851	**2.95E-05**
Treatment	1	11.874	11.874	1.8856	0.1913
Sex:Treatment	1	10.631	10.631	1.6882	0.2148
Residuals	14	88.159	6.297		

Table 14. Two-way ANOVA results testing the factors and interaction of sex and treatment on Unknown 5. Significant p-values are bolded.

	Df	Sum Sq	Mean Sq	F Value	Pr(>F)
Sex	1	70.172	70.172	36.9153	**2.86E-05**
Treatment	1	0.717	0.717	0.3771	0.549
Sex:Treatment	1	0.117	0.117	0.0616	0.8077
Residuals	14	26.613	1.901		

Table 15. Two-way ANOVA results testing the factors and interaction of sex and treatment on 2MeC26. Significant p-values are bolded.

	Df	Sum Sq	Mean Sq	F Value	Pr(>F)
Sex	1	5.112	5.1122	0.7155	0.41185
Treatment	1	30.899	30.899	4.3245	0.05643
Sex:Treatment	1	7.393	7.3935	1.0348	0.3263
Residuals	14	100.032	7.1451		

Table 16. Two-way ANOVA results testing the factors and interaction of sex and treatment on 11,13MeC27. Significant p-values are bolded.

	Df	Sum Sq	Mean Sq	F Value	Pr(>F)
Sex	1	25.1064	25.1064	13.5621	**0.00246**
Treatment	1	2.1742	2.1742	1.1745	0.29679
Sex:Treatment	1	10.9682	10.9682	5.9249	**0.02891**
Residuals	14	25.917	1.8512		

Table 17. Two-way ANOVA results testing the factors and interaction of sex and treatment on 2MeC28. Significant p-values are bolded.

	Df	Sum Sq	Mean Sq	F Value	Pr(>F)
Sex	1	11.686	11.686	2.6298	0.127169
Treatment	1	73.833	73.833	16.616	**0.001133**
Sex:Treatment	1	61.527	61.527	13.8467	**0.00228**
Residuals	14	62.209	4.443		

Table 18. Tukey's pairwise comparisons the factors and interaction of sex and treatment on 11,13MeC25. Male:Control-Female:Antibiotic and Female:Control-Male:Antibiotic comparisons are not shown because these comparisons are not relevant to questions addressed in this study. Significant p-values are bolded.

		diff	lwr	upr	p adj
Sex	Male-Female	-9.956	-13.41374	-6.498255	**2.41E-05**
Treatment	Control-Antibiotic	-3.0501	-6.507845	0.4076445	0.0793675
Sex:Treatment	Male:Antibiotic-Female:Antibiotic	-13.1425	-19.5191452	-6.7658548	**0.0001726**
	Female:Control-Female:Antibiotic	-6.535	-13.5202648	0.4502648	0.0702341
	Male:Control-Male:Antibiotic	-0.2025	-6.5791452	6.1741452	0.9997039
	Male:Control-Female:Control	-6.81	-13.7952648	0.1752648	0.0571204

Table 19. Tukey's pairwise comparisons the factors and interaction of sex and treatment on Unknown 1. Male:Control-Female:Antibiotic and Female:Control-Male:Antibiotic comparisons are not shown because these comparisons are not relevant to questions addressed in this study. Significant p-values are bolded.

		diff	lwr	upr	p adj
Sex	Male-Female	-4.36675	-6.763178	-1.970322	**0.0015761**
Treatment	Control-Antibiotic	-2.385675	-4.782103	0.01075276	0.0509031
Sex:Treatment	Male:Antibiotic-Female:Antibiotic	-0.7475	-5.166904	3.671904	0.9596792
	Female:Control-Female:Antibiotic	2.2225	-2.618714	7.063714	0.5576087
	Male:Control-Male:Antibiotic	-6.27	-10.689404	-1.850596	**0.0050431**
	Male:Control-Female:Control	-9.24	-14.081214	-4.398786	**0.0003711**

Table 20. Tukey's pairwise comparisons the factors and interaction of sex and treatment on Unknown 2. Male:Control-Female:Antibiotic and Female:Control-Male:Antibiotic comparisons are not shown because these comparisons are not relevant to questions addressed in this study. Significant p-values are bolded.

		diff	lwr	upr	p adj
Sex	Male-Female	0.0825	-1.011525	1.176525	0.8738236
Treatment	Control-Antibiotic	0.79425	-0.2997754	1.888275	0.1417612
Sex:Treatment	Male:Antibiotic-Female:Antibiotic	1.09	-0.9275613	3.107561	0.42536
	Female:Control-Female:Antibiotic	1.915	-0.2951276	4.125128	0.1001369
	Male:Control-Male:Antibiotic	-0.125	-2.1425613	1.892561	0.9978247
	Male:Control-Female:Control	-0.95	-3.1601276	1.260128	0.6076192

Table 21. Tukey's pairwise comparisons the factors and interaction of sex and treatment on Unknown 3. Male:Control-Female:Antibiotic and Female:Control-Male:Antibiotic comparisons are not shown because these comparisons are not relevant to questions addressed in this study. Significant p-values are bolded.

		diff	lwr	upr	p adj
Sex	Male-Female	2.7405	1.878684	3.602316	**8.30E-06**
Treatment	Control-Antibiotic	-0.11295	-0.9747661	0.7488661	0.7827479
Sex:Treatment	Male:Antibiotic-Female:Antibiotic	3.2158333	1.6265039	4.8051628	**0.000208**
	Female:Control-Female:Antibiotic	0.47	-1.2710232	2.2110232	0.8601707
	Male:Control-Male:Antibiotic	-0.6008333	-2.1901628	0.9884961	0.6959935
	Male:Control-Female:Control	2.145	0.4039768	3.8860232	**0.0141276**

Table 22. Tukey's pairwise comparisons the factors and interaction of sex and treatment on Unknown 4. Male:Control-Female:Antibiotic and Female:Control-Male:Antibiotic comparisons are not shown because these comparisons are not relevant to questions addressed in this study. Significant p-values are bolded.

		diff	lwr	upr	p adj
Sex	Male-Female	7.2095	4.656536	9.762464	**2.95E-05**
Treatment	Control-Antibiotic	-1.6263	-4.179264	0.9266636	0.1933957
Sex:Treatment	Male:Antibiotic-Female:Antibiotic	8.464167	3.7560852	13.172248	**0.0006575**
	Female:Control-Female:Antibiotic	0.06	-5.0974449	5.217445	0.9999854
	Male:Control-Male:Antibiotic	-3.061667	-7.7697482	1.646415	0.2758294
	Male:Control-Female:Control	5.3425	0.1850551	10.499945	**0.0412635**

Table 23. Tukey's pairwise comparisons the factors and interaction of sex and treatment on Unknown 5. Male:Control-Female:Antibiotic and Female:Control-Male:Antibiotic comparisons are not shown because these comparisons are not relevant to questions addressed in this study. Significant p-values are bolded.

		diff	lwr	upr	p adj
Sex	Male-Female	3.9735	2.570834	5.376166	**2.86E-05**
Treatment	Control-Antibiotic	0.3996	-1.003066	1.802266	0.5509789
Sex:Treatment	Male:Antibiotic-Female:Antibiotic	3.865	1.278254	6.451746	**0.0033359**
	Female:Control-Female:Antibiotic	0.225	-2.608638	3.058638	0.9954639
	Male:Control-Male:Antibiotic	0.5525	-2.034246	3.139246	0.9237047
	Male:Control-Female:Control	4.1925	1.358862	7.026138	**0.0036132**

Table 24. Tukey's pairwise comparisons the factors and interaction of sex and treatment on 2MeC26. Male:Control-Female:Antibiotic and Female:Control-Male:Antibiotic comparisons are not shown because these comparisons are not relevant to questions addressed in this study. Significant p-values are bolded.

		diff	lwr	upr	p adj
Sex	Male-Female	1.0725	-1.646945	3.791945	0.4118525
Treatment	Control-Antibiotic	2.6235	-0.09594473	5.342945	0.0575208
Sex:Treatment	Male:Antibiotic-Female:Antibiotic	2.520833	-2.494267	7.535933	0.4849355
	Female:Control-Female:Antibiotic	4.07	-1.423767	9.563767	0.1843967
	Male:Control-Male:Antibiotic	1.466667	-3.548433	6.481767	0.829837
	Male:Control-Female:Control	-0.0825	-5.576267	5.411267	0.9999686

Table 25. Tukey's pairwise comparisons the factors and interaction of sex and treatment on 11,13MeC27. Male:Control-Female:Antibiotic and Female:Control-Male:Antibiotic comparisons are not shown because these comparisons are not relevant to questions addressed in this study. Significant p-values are bolded.

		diff	lwr	upr	p adj
Sex	Male-Female	-2.37675	-3.760966	-0.9925343	**0.0024603**
Treatment	Control-Antibiotic	-0.695925	-2.080141	0.6882907	0.2991255
Sex:Treatment	Male:Antibiotic-Female:Antibiotic	-3.8883333	-6.441053	-1.3356137	**0.0028471**
	Female:Control-Female:Antibiotic	-2.4325	-5.228864	0.3638643	0.0984234
	Male:Control-Male:Antibiotic	0.7383333	-1.814386	3.291053	0.8343281
	Male:Control-Female:Control	-0.7175	-3.513864	2.0788643	0.8769391

Table 26. Tukey's pairwise comparisons the factors and interaction of sex and treatment on 2Mec28. Male:Control-Female:Antibiotic and Female:Control-Male:Antibiotic comparisons are not shown because these comparisons are not relevant to questions addressed in this study. Significant p-values are bolded.

		diff	lwr	upr	p adj
Sex	Male-Female	1.6215	-0.523051	3.766051	0.1271689
Treatment	Control-Antibiotic	4.0554	1.910849	6.199951	**0.0011797**
Sex:Treatment	Male:Antibiotic-Female:Antibiotic	-1.3825	-5.337402	2.572402	0.7430054
	Female:Control-Female:Antibiotic	0	-4.332378	4.332378	1
	Male:Control-Male:Antibiotic	7.51	3.555098	11.464902	**0.00039**
	Male:Control-Female:Control	6.1275	1.795122	10.459878	**0.0051664**

Table 27. Principal Component Analysis (PCA) for each D. sproati sample analyzed for CHC production.

	PC1	PC2	PC3	PC4	PC5	PC6	PC7	PC8	PC9
Control Male 1	1.701741	5.586216	2.87965	4.051169	4.717201	11.31314	6.243181	0.445183	33.32763
Control Male 2	6.907947	2.900605	8.513999	5.219978	3.123374	10.32303	4.571086	1.646558	33.32975
Control Male 3	-1.77049	6.181229	6.685612	-0.56721	6.848432	12.27616	5.392077	0.64901	33.32772
Control Male 4	4.777692	0.868306	5.951149	5.735041	6.558861	11.70714	4.926663	0.490494	33.3272
Antibiotic Male 1	-0.07225	-6.72675	7.108058	2.901824	7.093445	10.88987	6.614399	1.114458	33.32823
Antibiotic Male 2	5.016395	-1.71033	6.55338	5.150574	7.579597	13.68057	4.813937	1.548115	33.32926
Antibiotic Male 3	6.991599	-9.91536	10.68093	2.608988	3.883119	13.50816	5.445564	0.437428	33.3297
Antibiotic Male 4	10.17217	-5.7121	7.74976	4.278961	5.905181	10.77462	6.486287	1.536316	33.32399
Antibiotic Male 5	5.739779	-7.1598	0.494991	2.04204	5.180655	11.91738	3.744243	1.037055	33.32488
Antibiotic Male 6	1.266213	-6.61077	2.771294	4.822853	6.484166	10.78569	5.728739	0.544456	33.33113
Control Female 1	17.60448	-2.34879	-4.37726	2.159198	4.716302	12.01982	6.138655	1.333678	33.32964
Control Female 2	13.43912	-2.26335	5.360349	5.601185	4.57852	11.97533	5.63595	-0.32022	33.32635
Control Female 3	9.962258	-3.70794	1.139093	4.75983	8.030634	10.90009	4.793989	0.421934	33.32874
Control Female 4	20.78447	-1.25876	2.30265	3.827654	5.60263	12.14179	5.668124	0.85125	33.32822
Antibiotic Female 1	14.35043	0.008722	6.055521	5.755688	6.650066	12.52466	5.764608	1.213222	33.32802
Antibiotic Female 2	21.06335	1.399638	5.694906	4.692712	6.739393	12.80034	5.437804	0.872736	33.32731

Antibiotic Female 3	22.27172	-0.95435	8.048583	2.262045	6.093777	12.52994	5.875682	0.829496	33.32845
Antibiotic Female 4	25.99018	-2.31984	9.720507	1.715776	6.813495	9.56348	4.778242	0.631302	33.32905
% Variation	64.2%	0.172244	0.125257	0.027354	0.015711	0.010679	0.004906	0.002268	2.75E-08

Table 28. Two-way ANOVA testing the factors and interaction of sex and treatment on PC1. Significant p-values are bolded.

	Df	Sum Sq	Mean Sq	F Value	Pr(>F)
Sex	1	884.88	884.88	49.2354	**6.08E-06**
Treatment	1	55.44	55.44	3.0846	0.1009
Sex:Treatment	1	13.54	13.54	0.7535	0.4
Residuals	14	251.61	17.97		

Table 29. Two-way ANOVA testing the factors and interaction of sex and treatment on PC2. Significant p-values are bolded.

	Df	Sum Sq	Mean Sq	F Value	Pr(>F)
Sex	1	2.839	2.839	0.6197	0.444297
Treatment	1	96.439	96.439	21.0464	**0.000422**
Sex:Treatment	1	160.201	160.201	34.9614	3.78E-05
Residuals	14	64.151	4.582		

Figures

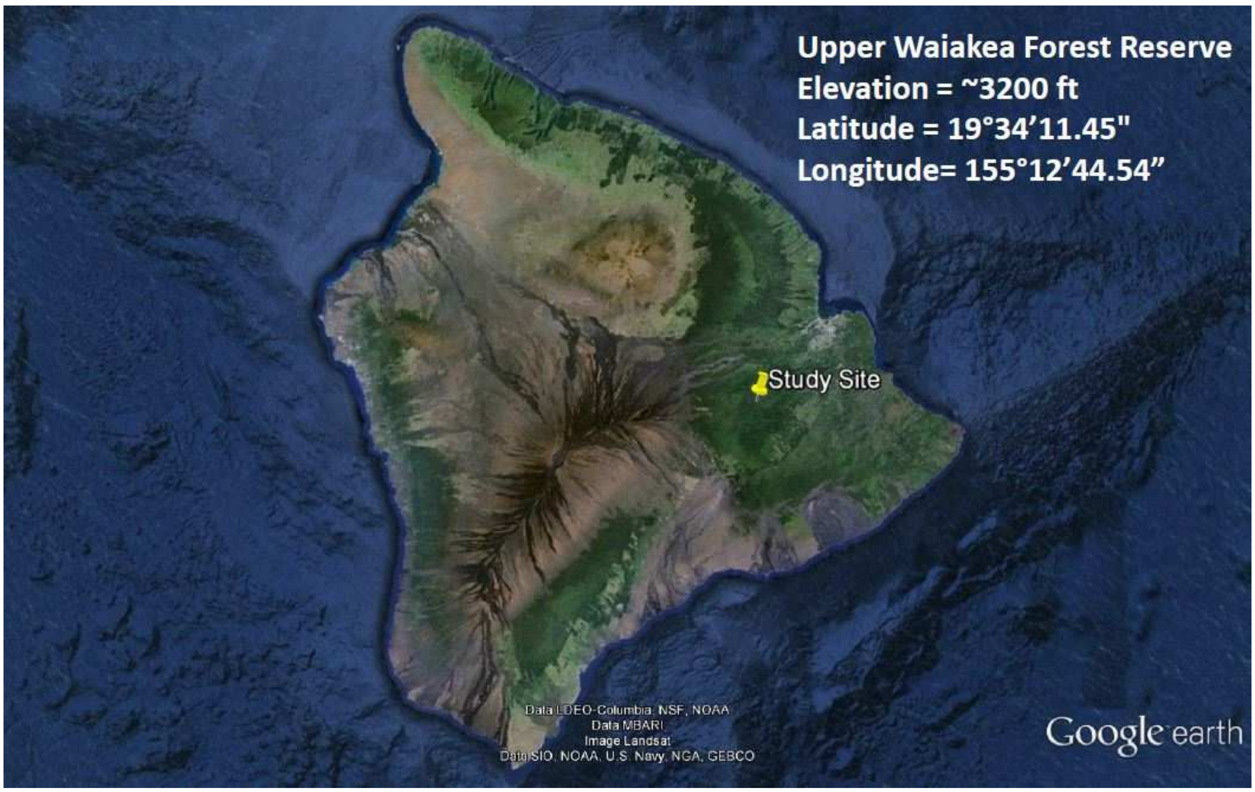

Figure 1. Field collection site.

Figure 2. Picture of multilure trap used to catch *Drosophila sproati*.

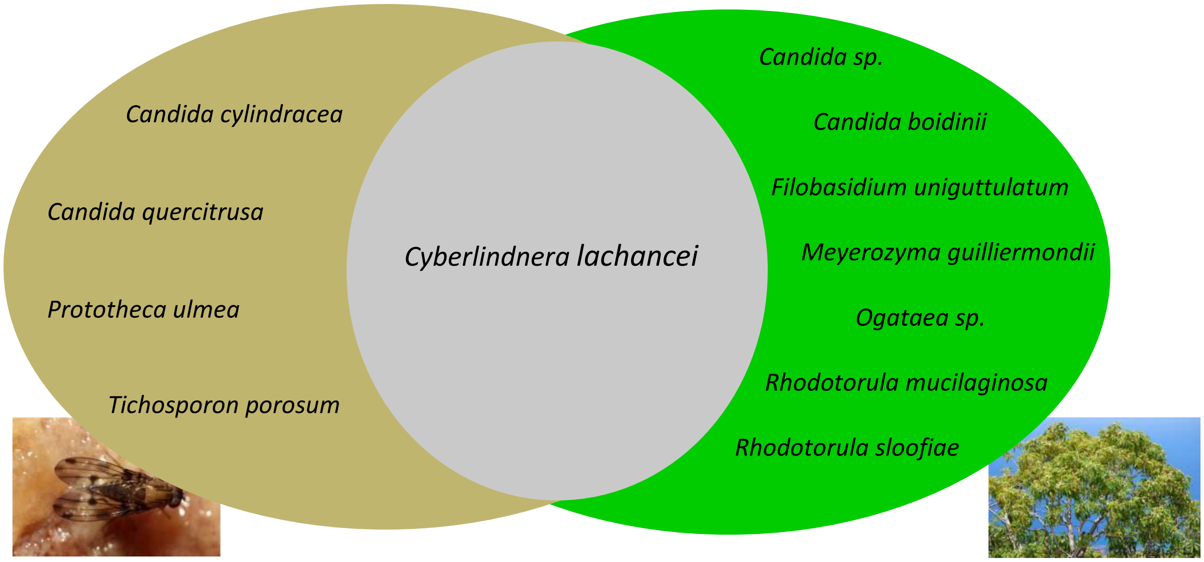

Figure 3. Venn diagram depicting yeast diversity results comparing *D. sproati* and its host plant *Cheirodendron trigynum*. Yeast species were identified by sequencing of the D1/D2 26S rRNA gene. The species shown in the brown section were found only on *D. sproati*. The species shown in the green section were found only on *Cheirodendron trigynum*. The middle section indicates the species that was found on both fly and host-plant, *Cyberlindnera lachancei*.

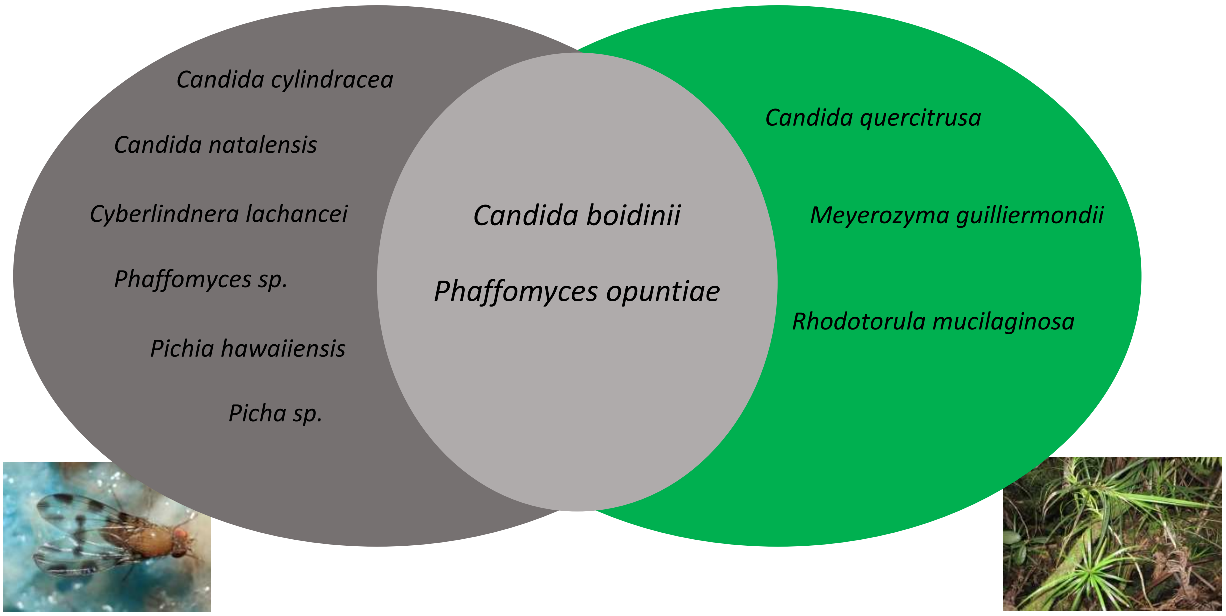

Figure 4. Venn diagram depicting yeast diversity results comparing *D. ochracea* and its host plant *Freycinetia arborea*. Yeast species were identified by sequencing of the D1/D2 26S rRNA gene. The species shown in the brown section were found only on *D. ochracea*. The species shown in the green section were found only on *Freycinetia arborea*. The section in the middle indicates species that was found on both fly and host-plant, *Candida boidinii* and *Phaffomyces opuntiae*.

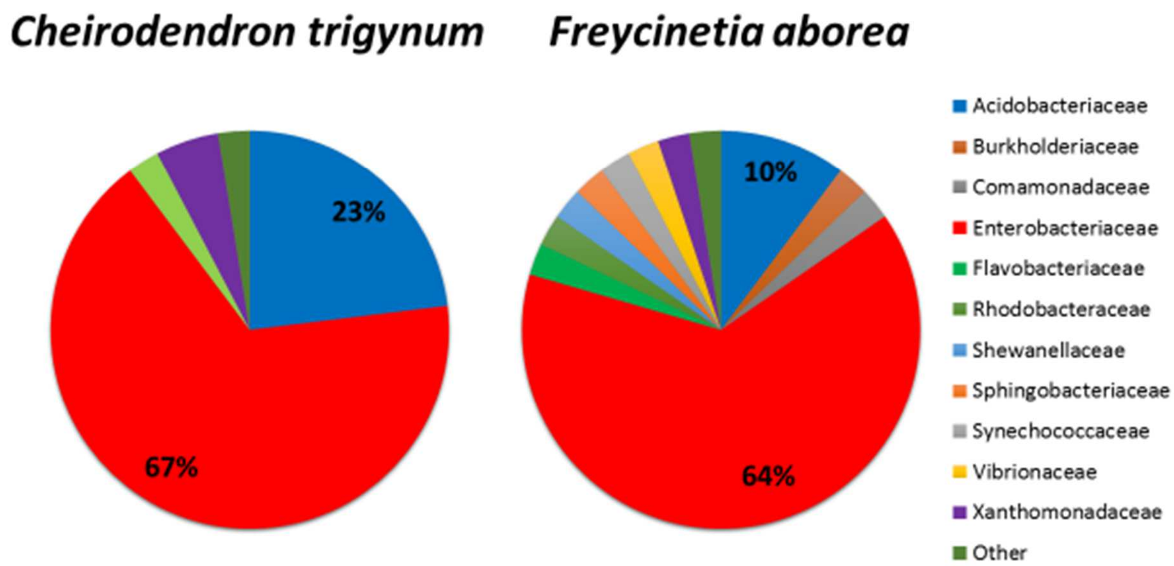

Figure 5. Pie charts display percent composition of the bacterial families of both host plants; *Cheirodendron trigynum* and *Freycinetia arborea*.

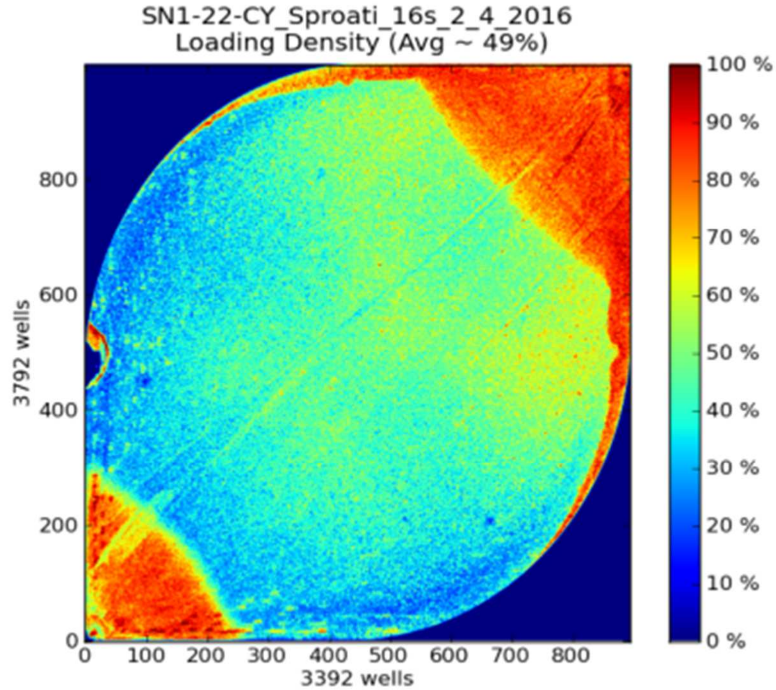

Figure 6. Ion Torrent 318v2 BC Chip loading density. ISP loading was 49%, 100% enrichment, 77% clonal, with a final library of 87% for a total of 3,719,939 reads.

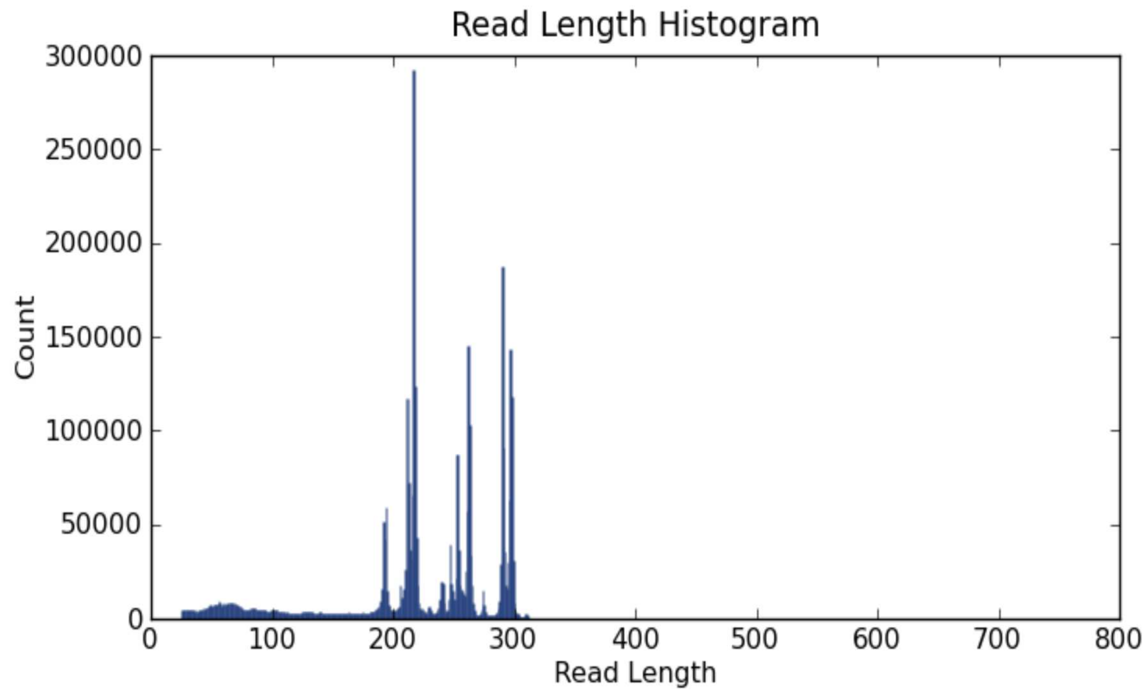

Figure 7. Histogram of total Ion Torrent PGM 16s reads showing the amplified product in base pairs (x-axis) by the number of sequences produced (y-axis).

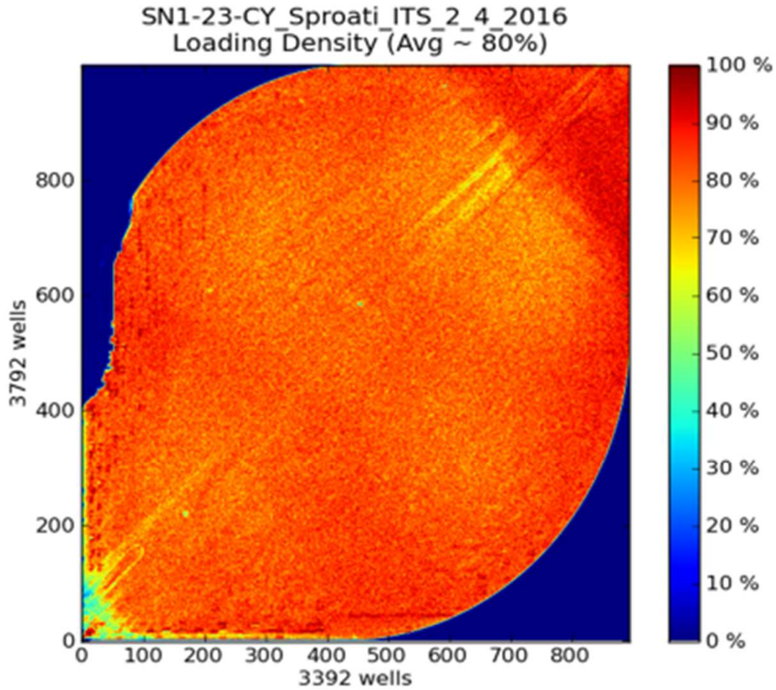

Figure 8. Ion Torrent 318v2 BC Chip loading density. ISP loading was 80%, 100% enrichment, 85% clonal, with a final library of 96% for a total of 7,352,309 reads.

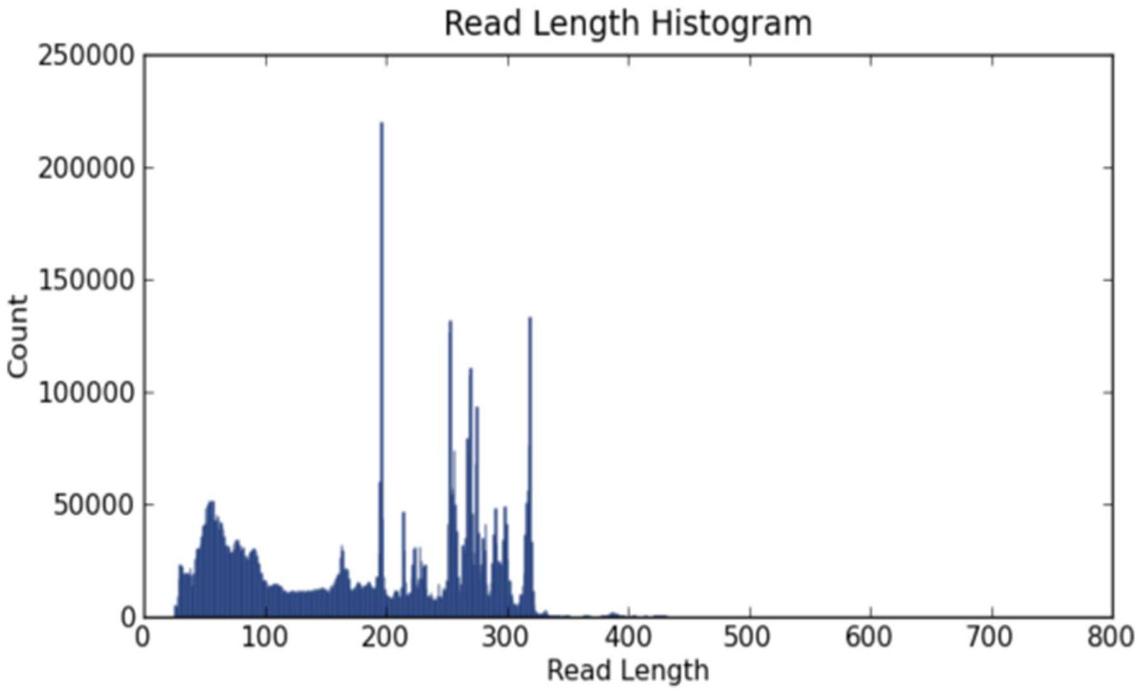

Figure 9. Histogram of total Ion Torrent PGM ITS1 reads showing the amplified product in base pairs (x-axis) by the number of sequences produced (y-axis).

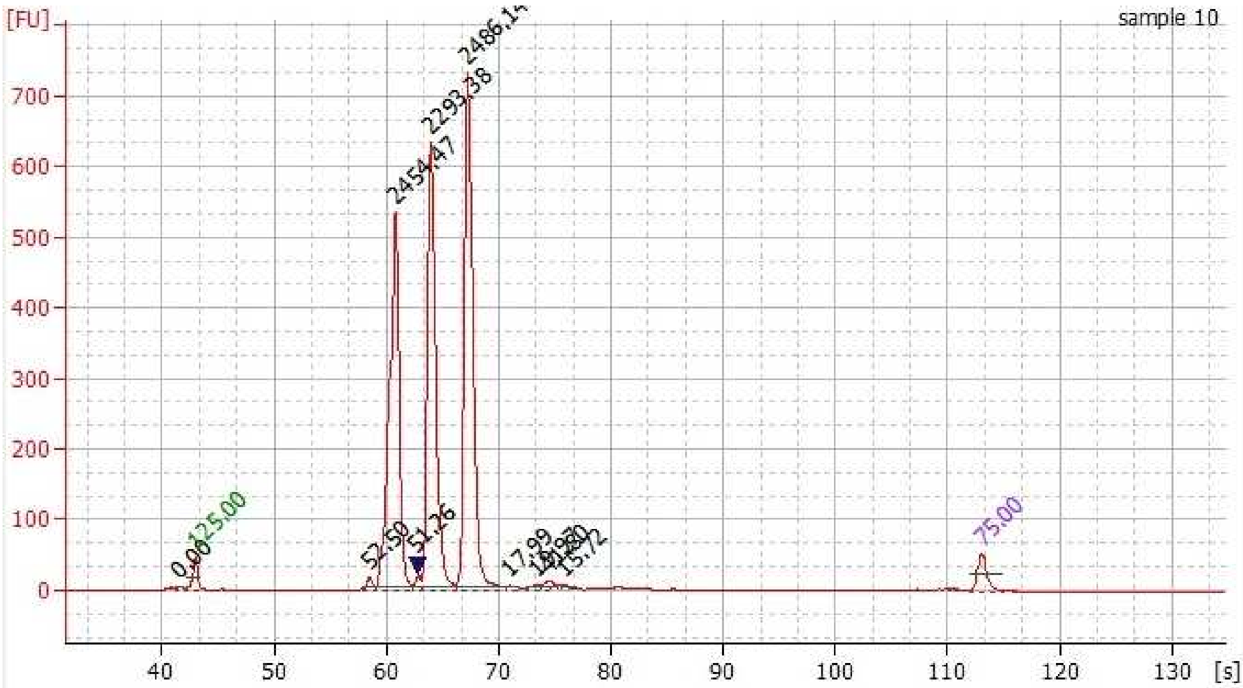

Figure 10. Example of amplified 16s hypervariable regions fragments run on a 2100 Agilent Bioanalyzer High Sensitivity kit.

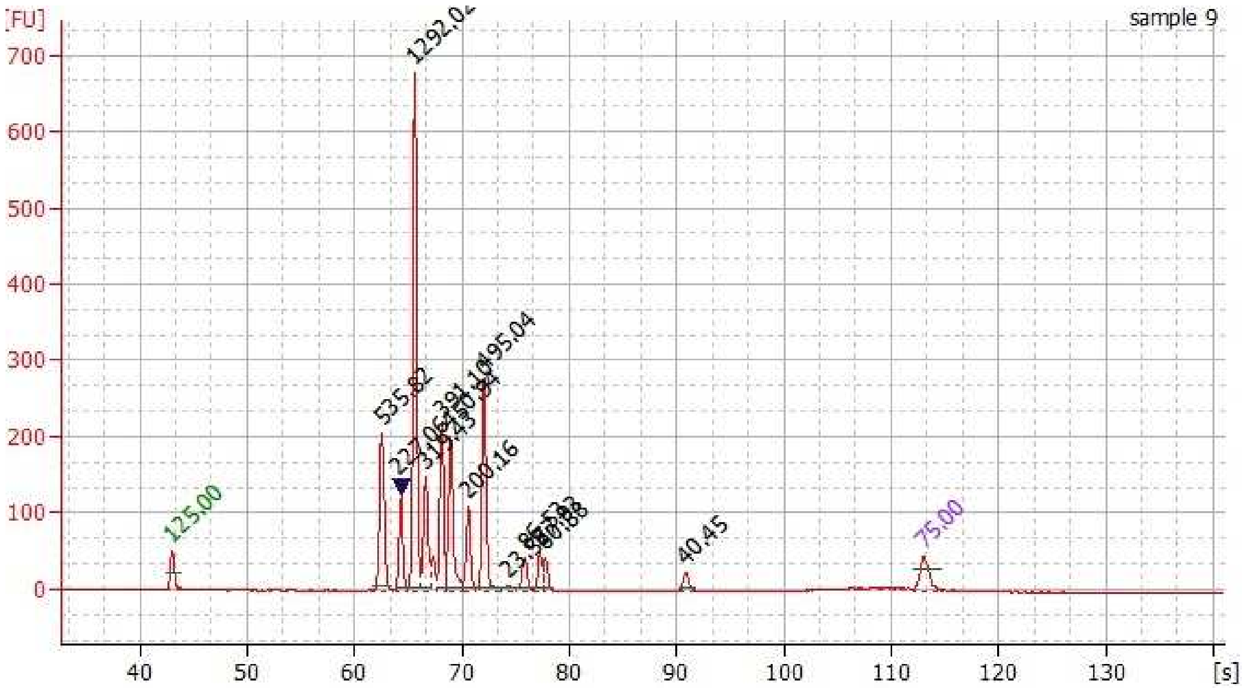

Figure 11. Example of amplified ITS1 fragments run on a 2100 Agilent Bioanalyzer High Sensitivity kit.

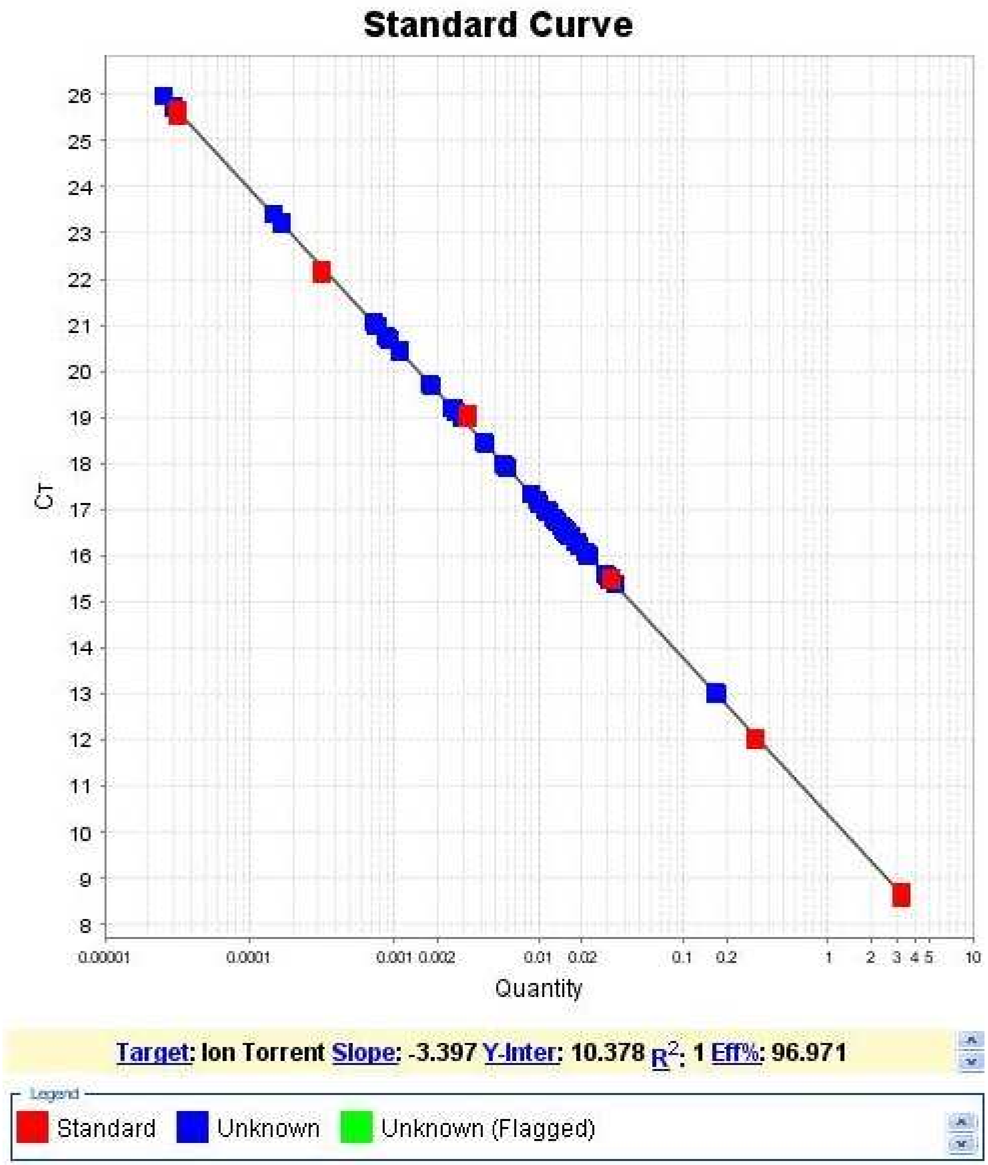

Figure 12. Example of qPCR standard curve used to determine optimal DNA loading for emulsion PCR. X-axis is quantify of DNA and Y-axis is temperature melting point. Red dots are standard, blue dots are the unknown DNA, and the green dots are flagged unknown DNA.

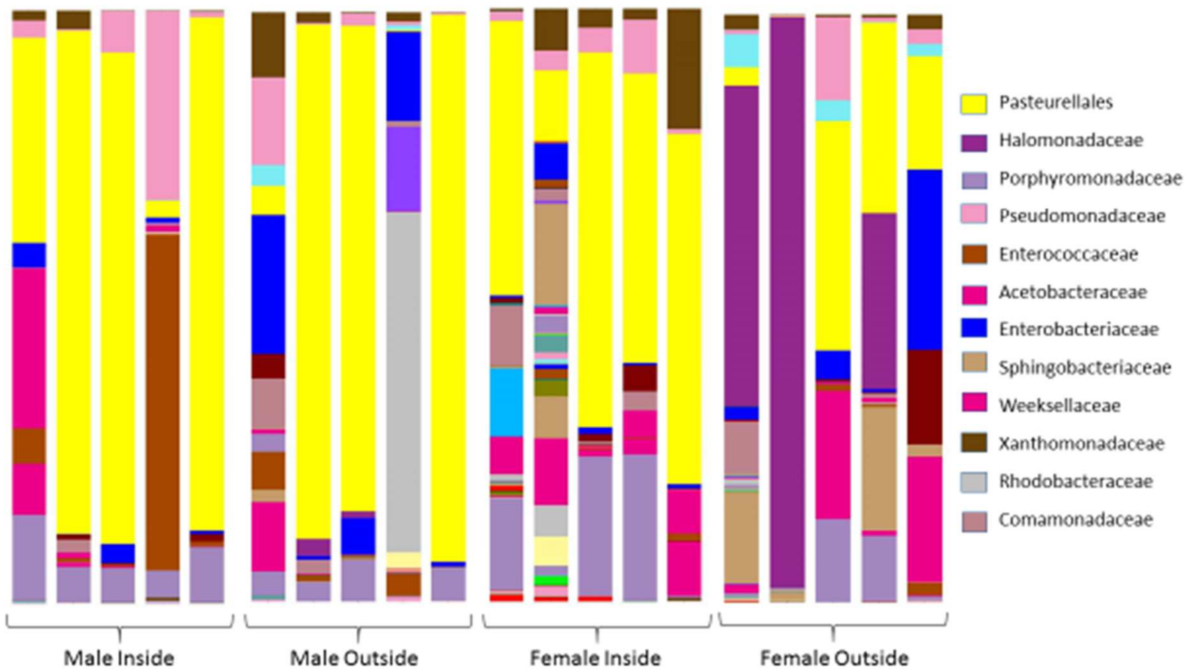

Figure 13. *Drosophila sproati* 16S metagenomic results. Bar chart illustrating family level taxon richness detected in each individual sample within the each group. Taxa shown in the legend consist of 95% of the OTUs found within our dataset.

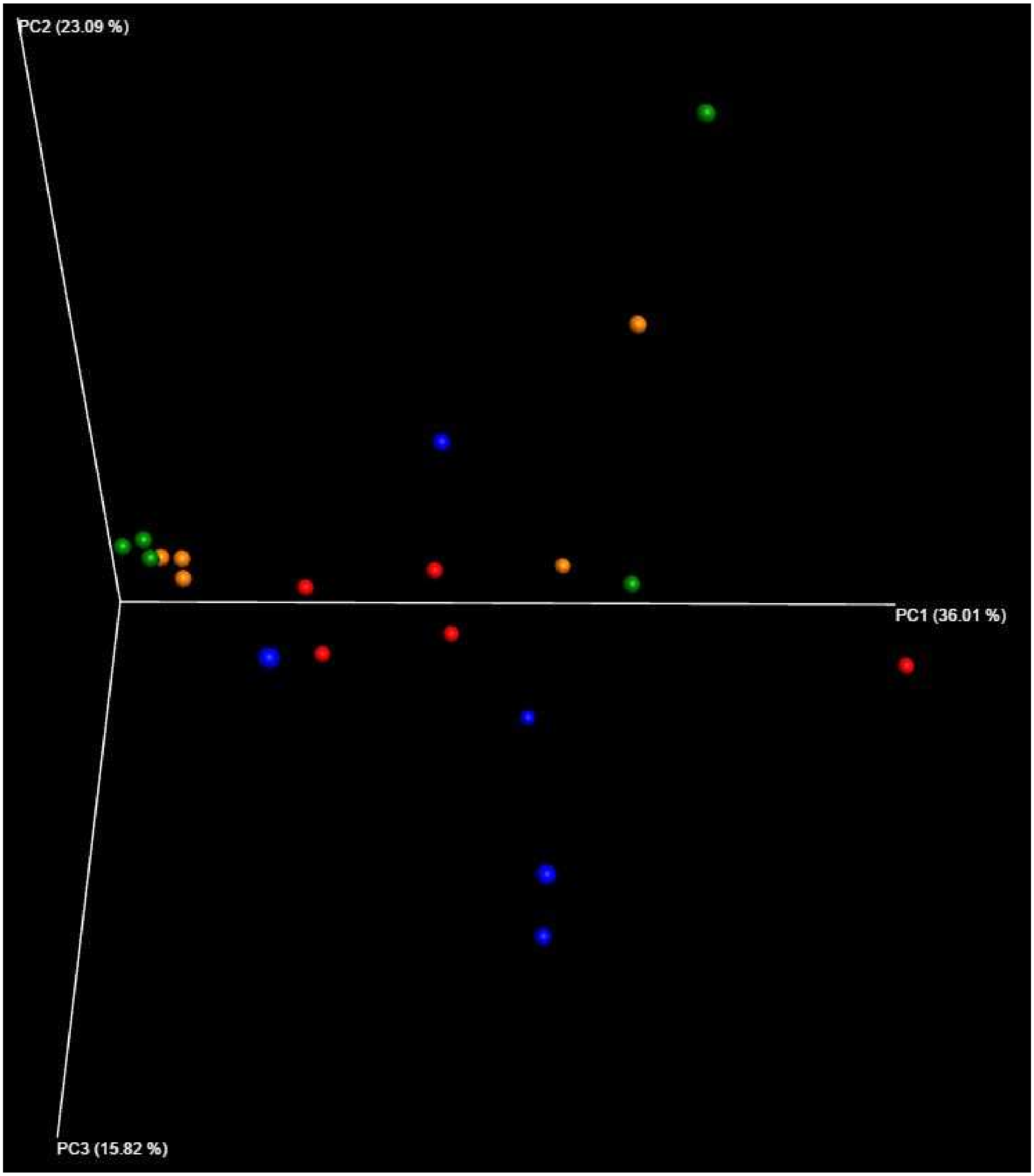

Figure 14. Weighted Unifrac PCoA plot illustrating the distance of *Drosophila sproati* microbial compositions accounting for abundance of observed. Male insides samples are shown in orange, male outside samples are shown in green, female inside samples are shown in red, and female outside samples are shown in blue.

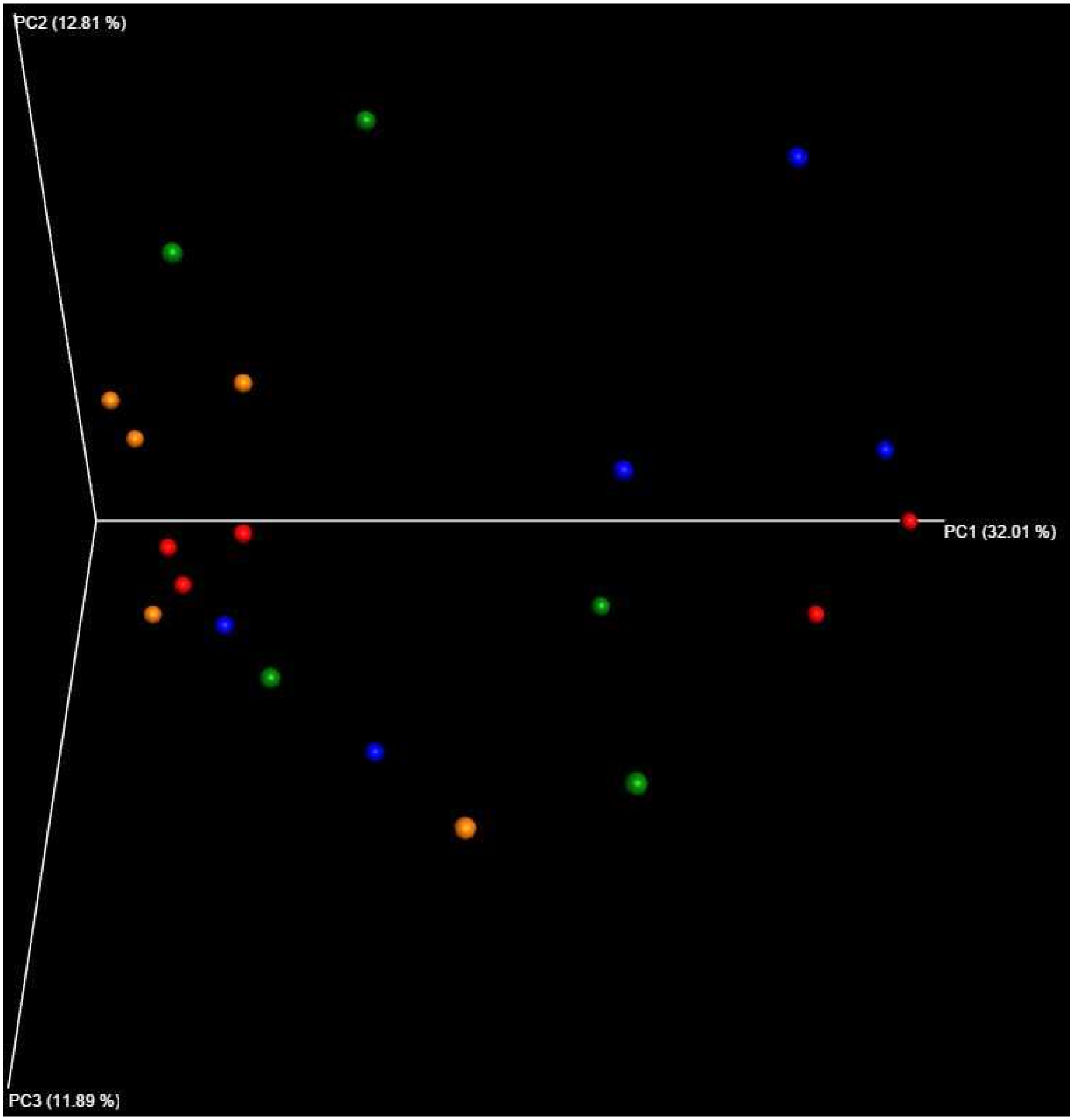

Figure 15. Unweighted Unifrac PCoA plot illustrating the distance of *Drosophila sproati* bacterial compositions based only on bacterial composition. Male insides samples are shown in orange, male outside samples are shown in green, female inside samples are shown in red, and female outside samples are shown in blue.

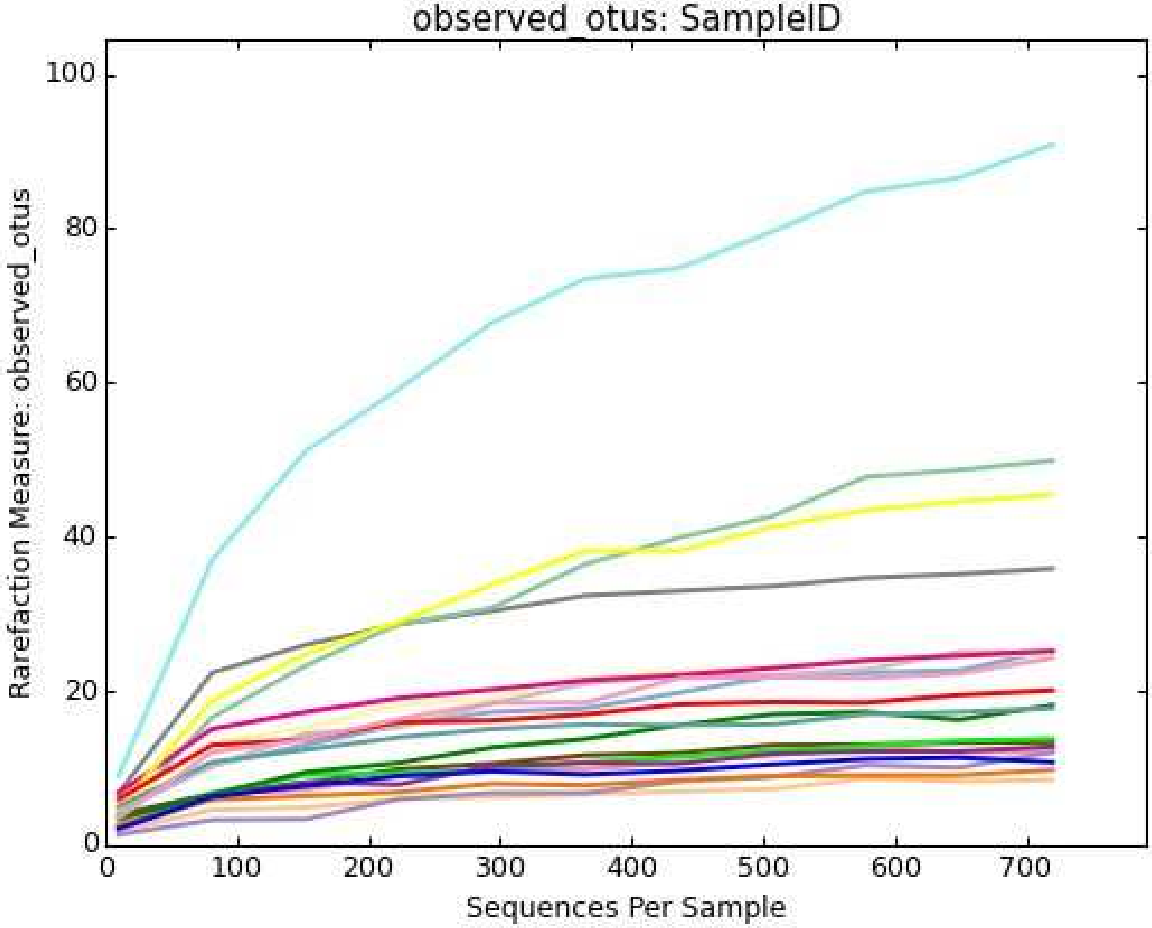

Figure 16. Rarefaction analysis of 16S our metagenomic results. Each individual sample within our dataset is shown with its own color. The outlier are from a female *Drosophila sproati* outside samples shown in light blue.

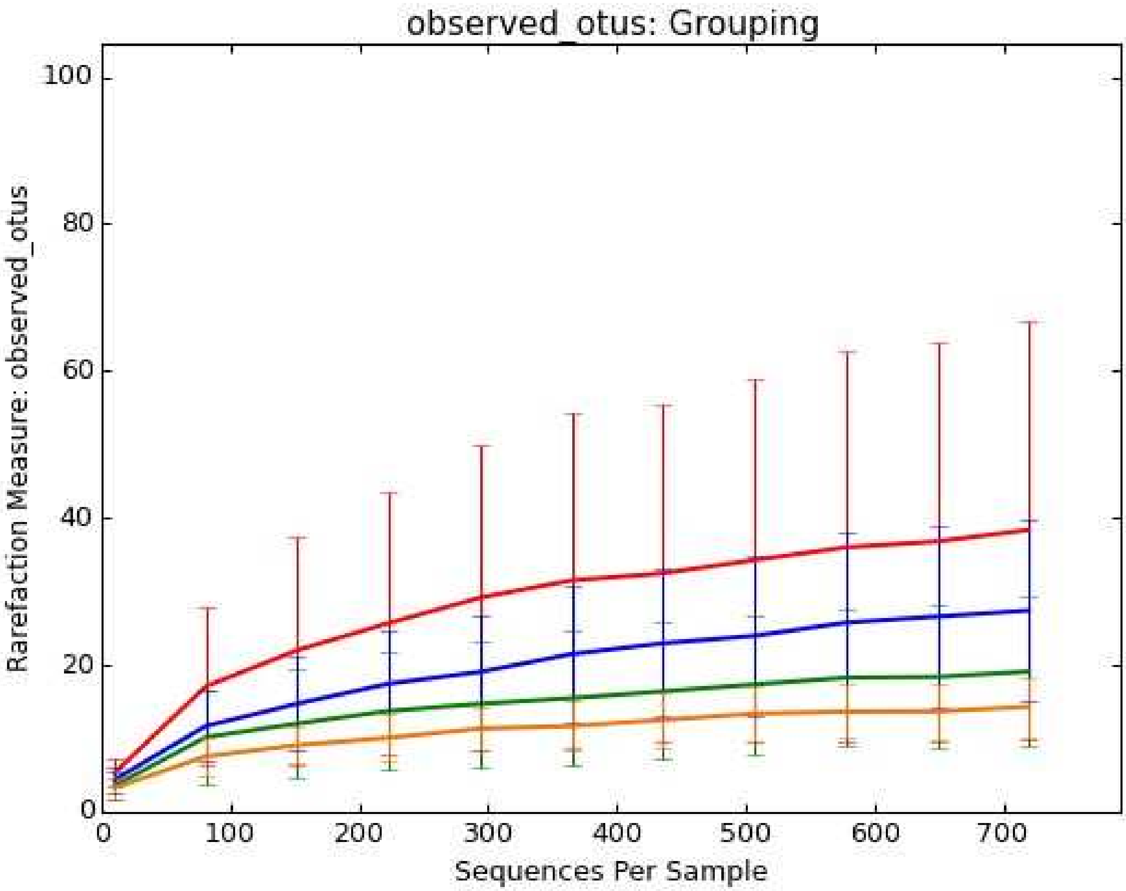

Figure 17. Rarefaction analysis of 16S our metagenomic results. Male inside is shown in orange, male outside is shown in green, female inside is shown in red, and female outside is shown in blue. Each sampling group is shown with error bars

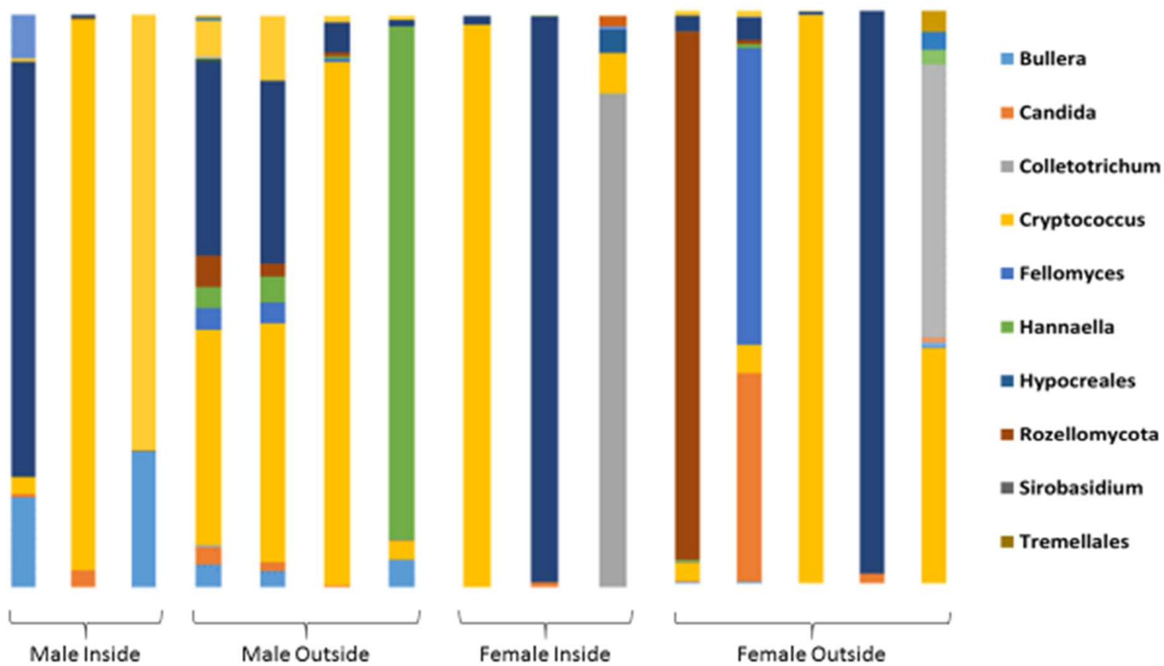

Figure 18. *Drosophila sproati* ITS1 metagenomic results. Bar chart illustrating taxon richness detected in each individual sample within the each group. All taxa found are shown in the bar chart, however only identified taxa are shown in the legend.

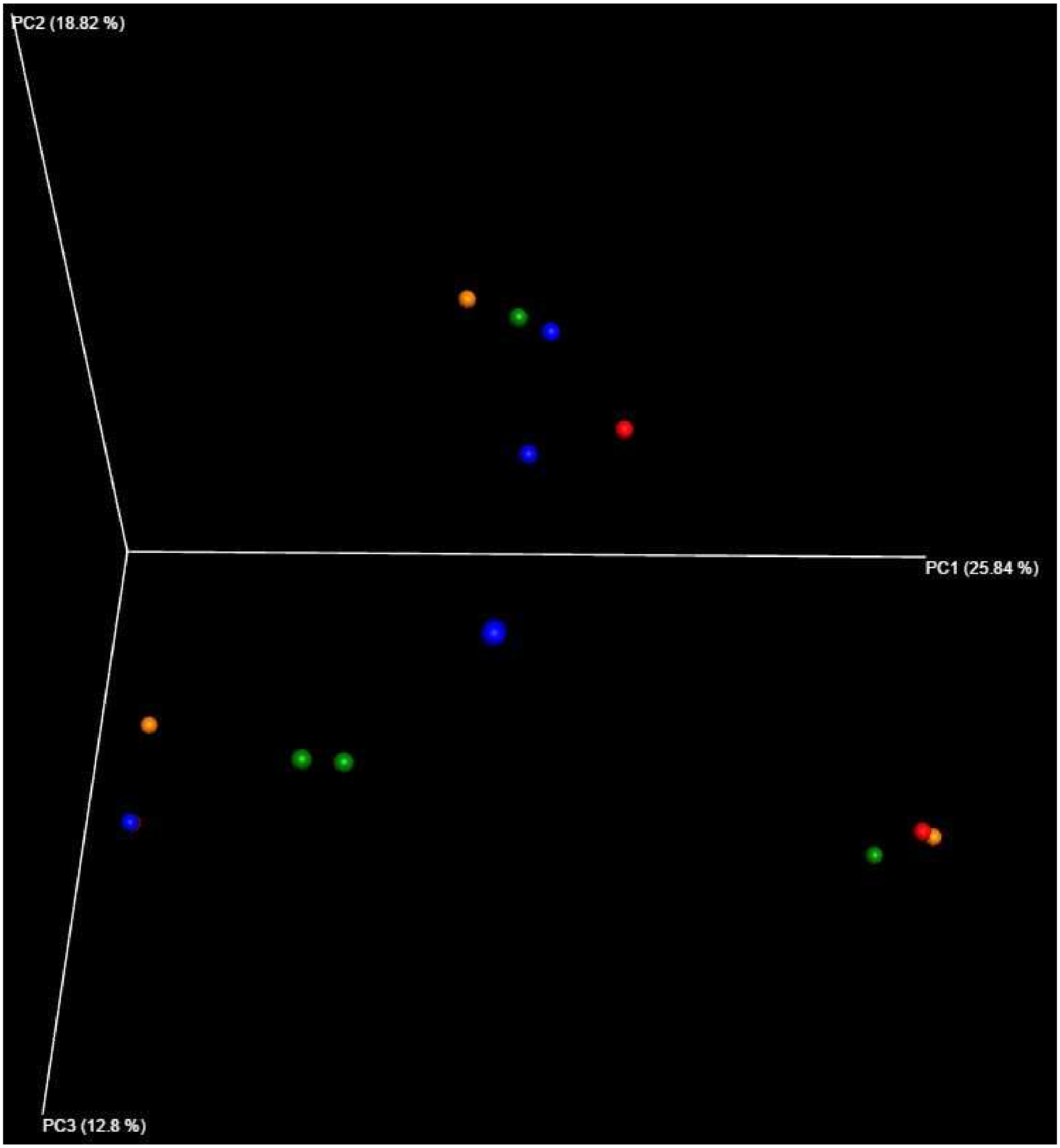

Figure 19. Bray-Curtis dissimilarity PCoA plot illustrating differences in fungal composition

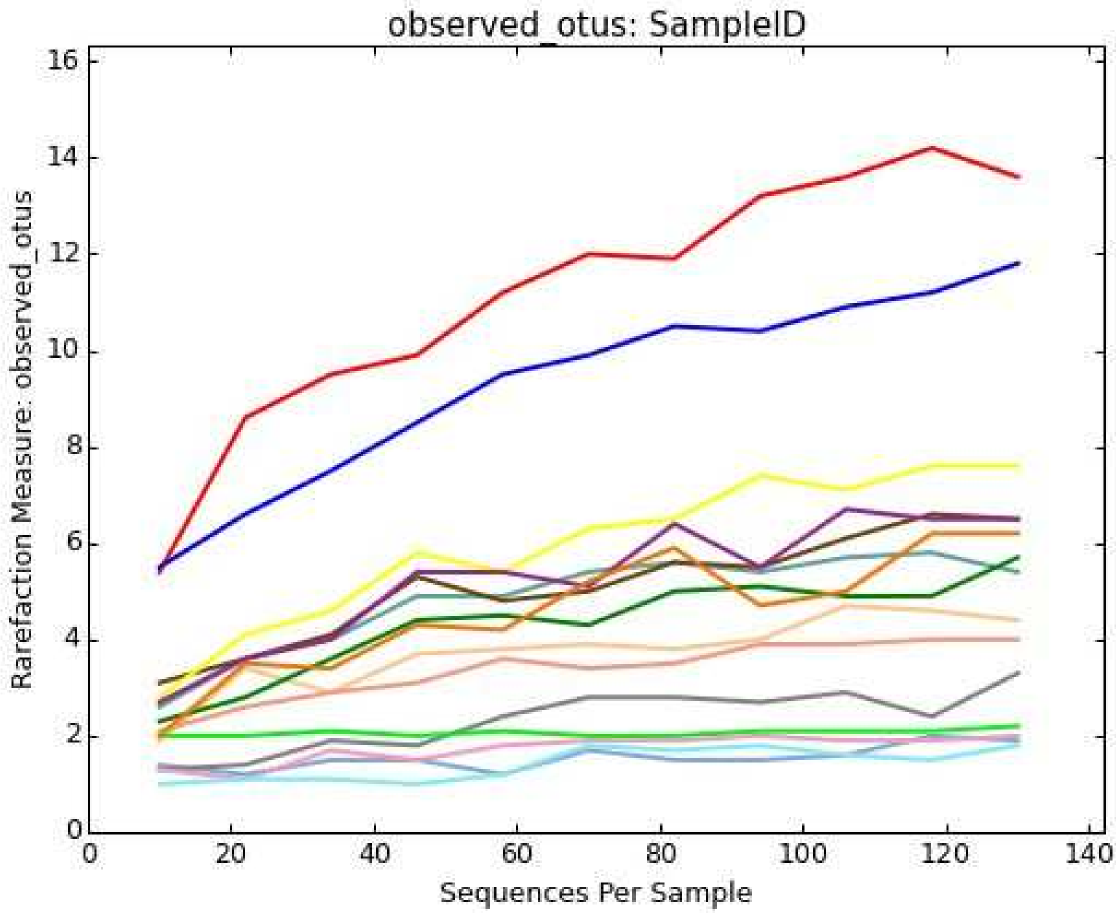

Figure 20. Rarefaction analysis of our ITS1 metagenomic results. Each individual sample within our dataset is shown with its own color. The outliers are from two male *Drosophila sproati* outside samples shown in red and blue.

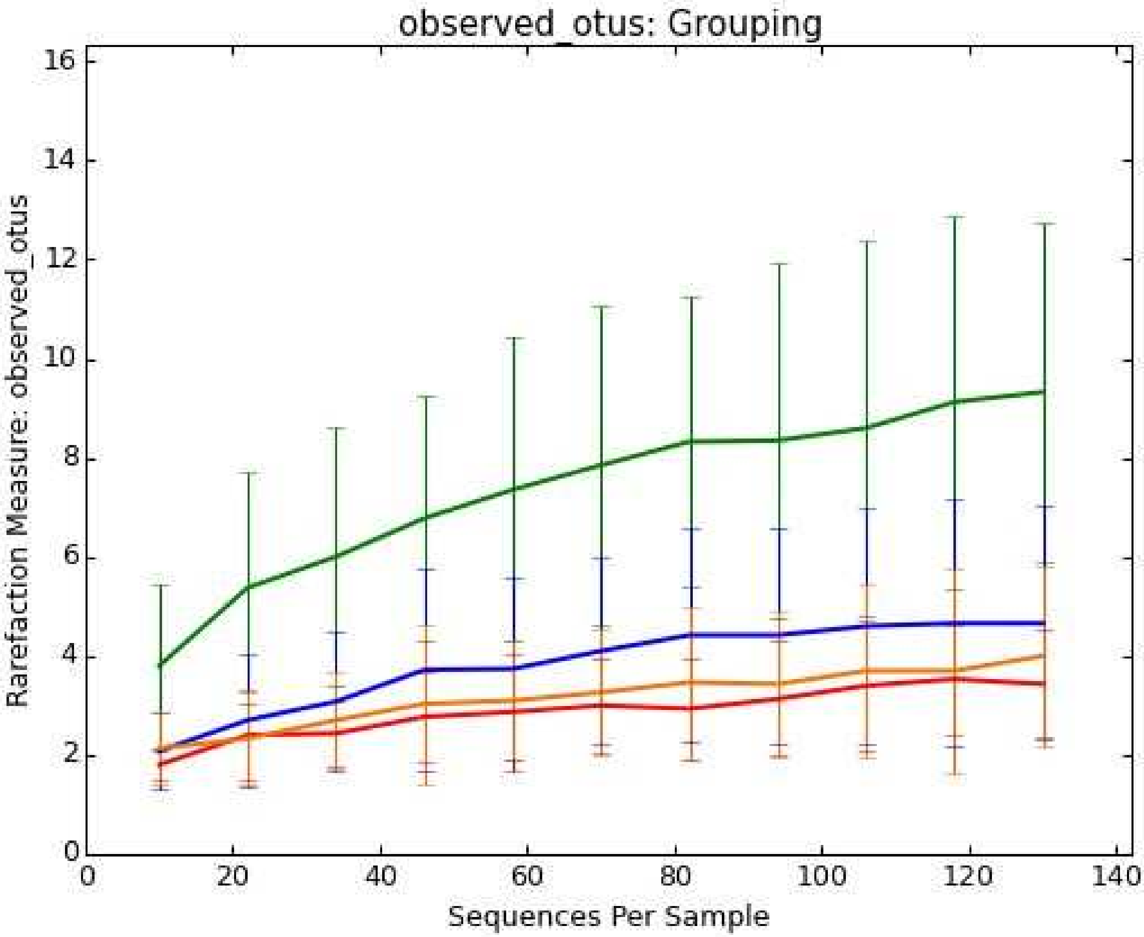

Figure 21. Rarefaction analysis of our ITS1 metagenomic results. Male inside is shown in orange, male outside is shown in green, female inside is shown in red, and female outside is shown in blue. Each sampling group is shown with error bars

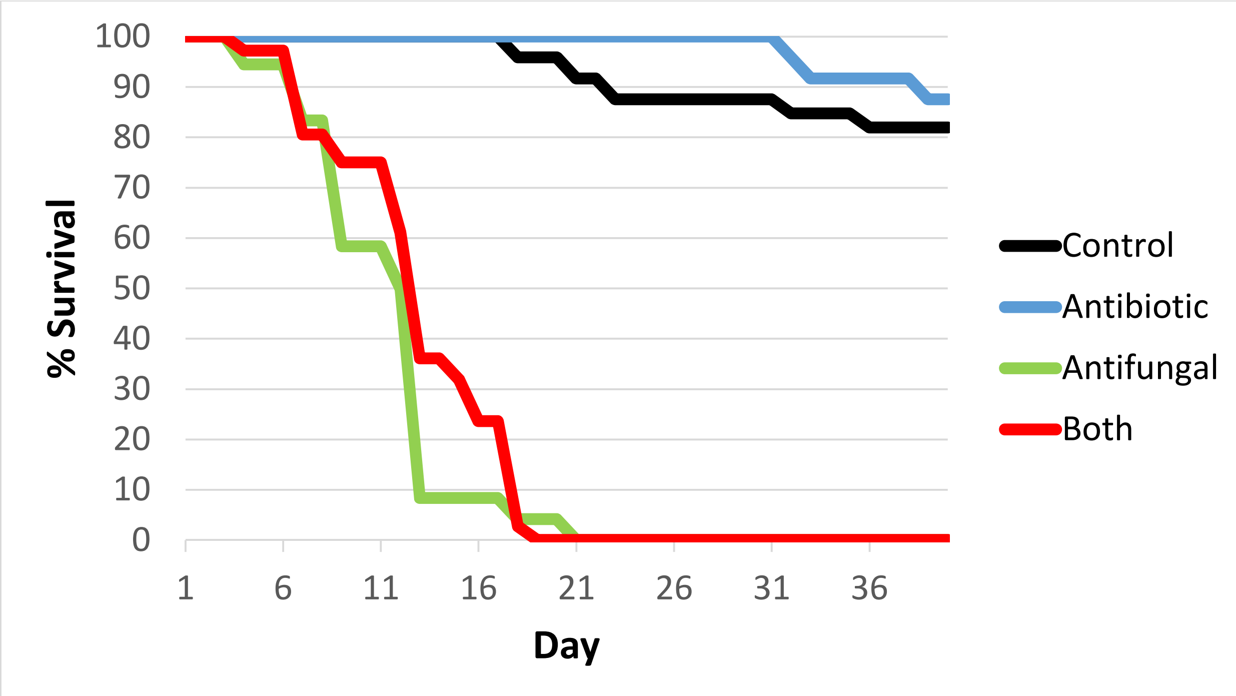

Figure 22. Mortality comparisons between treatments. Cox proportional hazard model (z = 6.44, p = 1.20e-10) and two-way nested ANOVA (F = 553.9, df = 3, p = 2.20e-16) show that treatment had a significant effect on mortality rate.

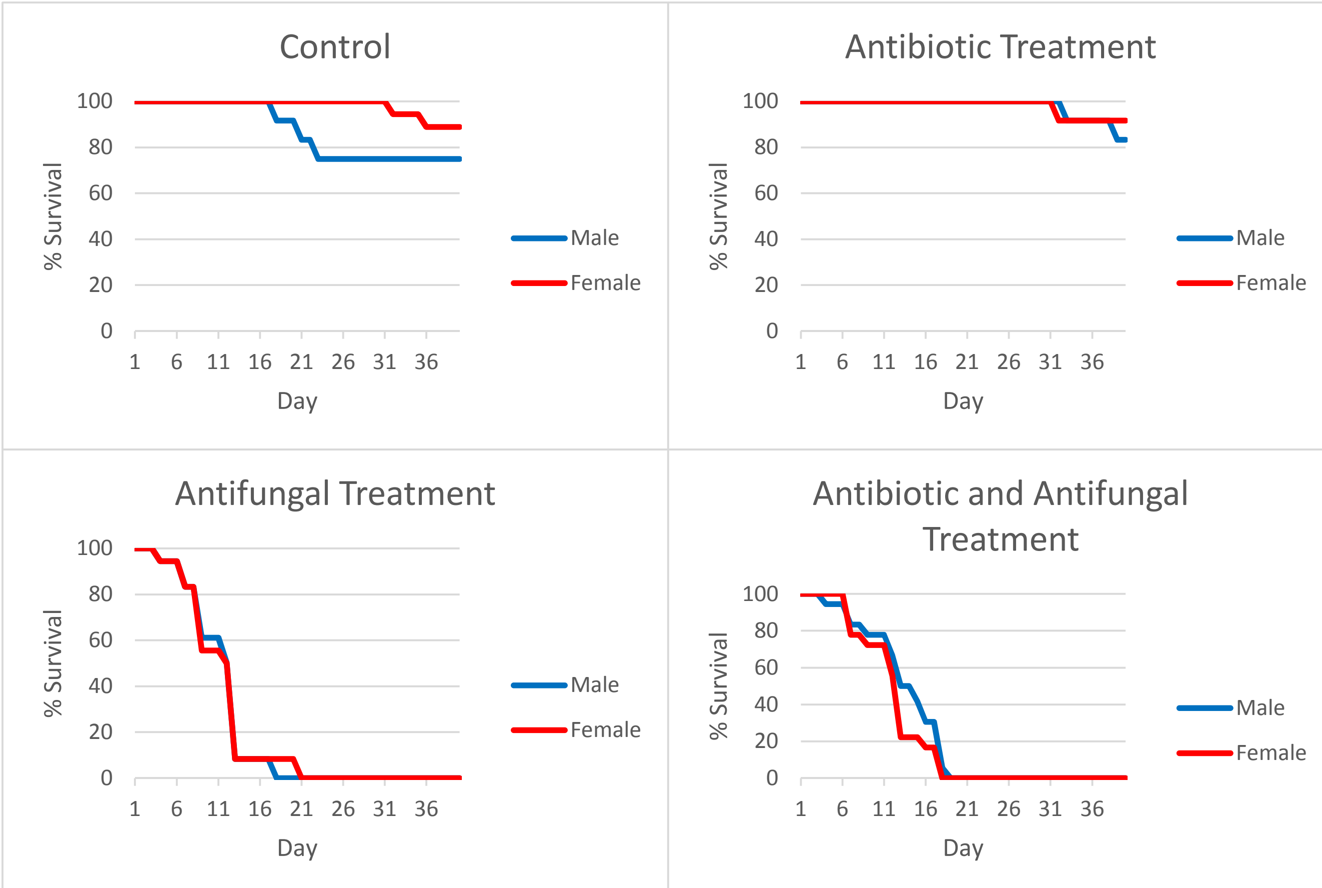

Figure 23. Comparison of mortality rates between male and female *D. sproati* within treatment groups. Cox proportional hazard model ($z = 1.67$, $p = 0.095$) and two-way nested ANOVA ($F = 0.5236$, $df = 1$, $p = 0.4712$) show that sex does not have a significant effect on mortality rate.

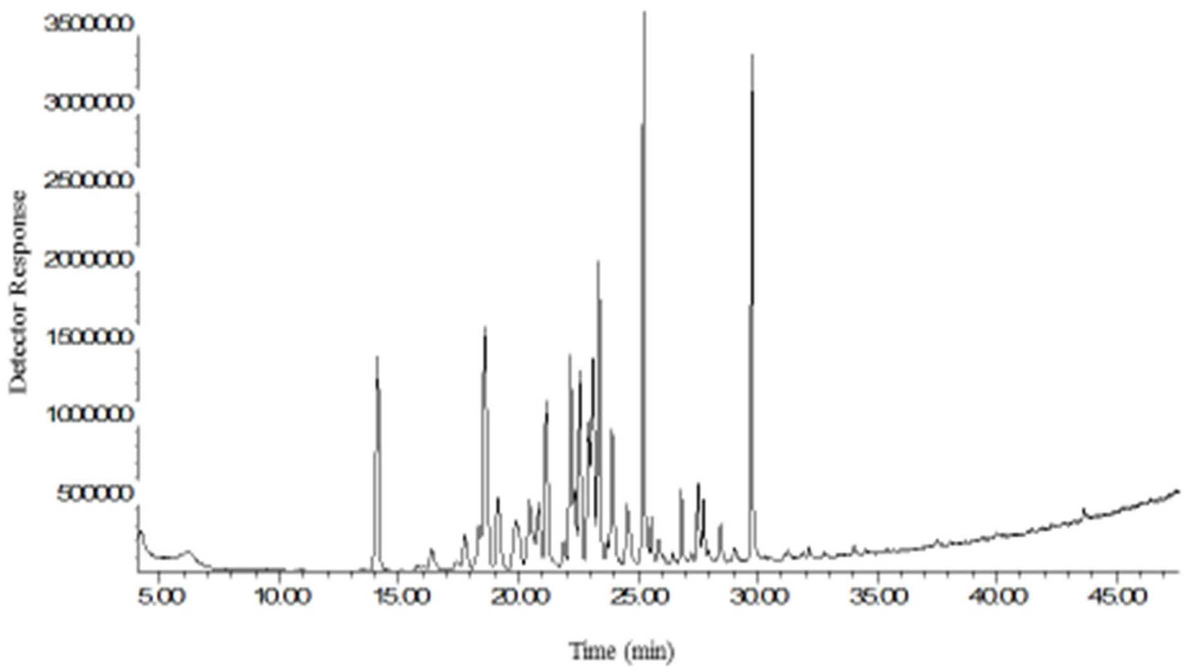

Figure 24. Male *Drosophila sproati* hydrocarbon profile.

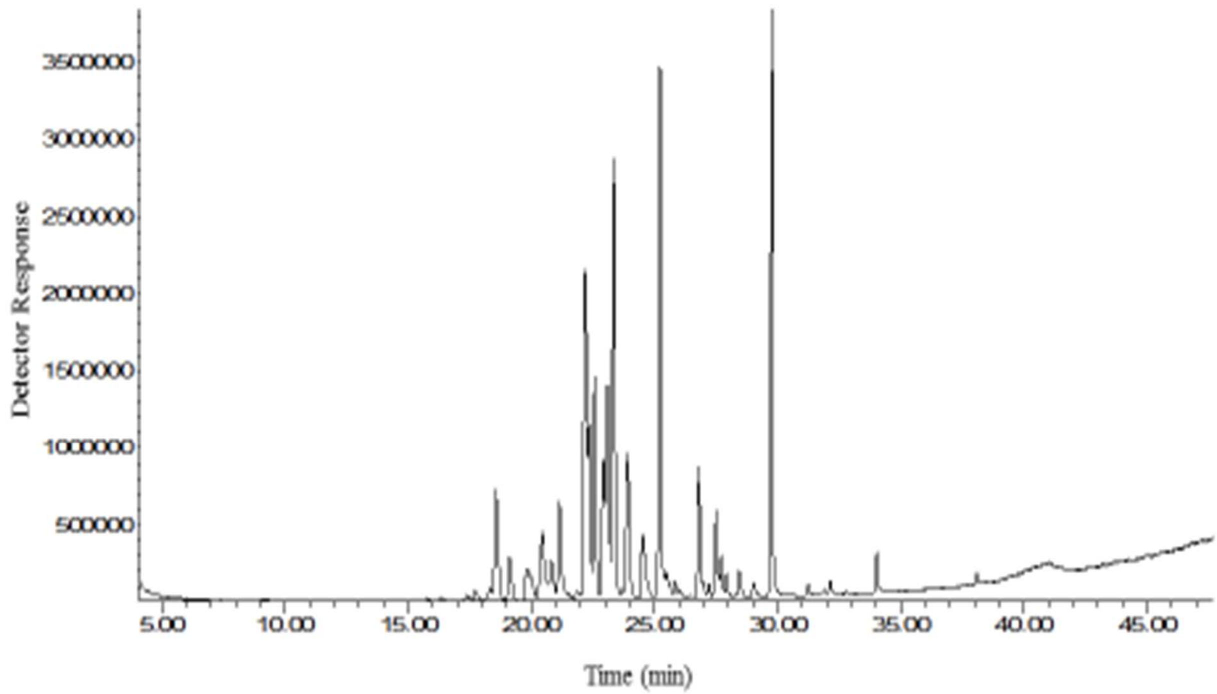

Figure 25. Female *Drosophila sproati* hydrocarbon profile.

Appendices

Appendix 1. Adapted Wheeler-Clayton Adult *Drosophila* Food Recipe
1) Mix 1g agar and 30mL dH20 together, microwave in Büchner flask for 1 minute at 100%.

2) Mix 3g powder mixture*, 24g banana baby food, and 15mL dH20 in a circular bowl, add to flask containing agar mixture and microwave for 2 minutes at 60%.

3) Stir and add 45mL dH20 and microwave 20% for 6 minutes.

4) Let cool and add 375uL propionic acid and 375uL 95% EtOH.

5) Aliquot into glass shell vials sufficient to fill approximately 1cm deep and refrigerate until needed. Use within 2 weeks to maintain freshness.
* powder mixture: mix equal parts by weight of wheat germ, high protein cereal, and Special K – blend, strain, and store in refrigerator/freezer.

Appendix 2. Larvae *Drosophila* Food Recipe
1) Mix 6g agar and 225mL dH20 and microwave for 2:22 at 100%.

2) Mix 60g cornmeal, 6.6g roasted soybean meal, and 7.5g baking yeast together and add 300mL dH20.

3) Add 3 spoonfuls of corn syrup and 1 spoonful of unsulfured molasses. Microwave 3 minutes at 50%, stop at 1:30 (halfway through) and mix. Continue microwaving for the remaining time, watching the mixture. As it rises, stop and mix. Do not let overflow.

4) Let cool and add 3mL propionic acid and 3mL 95% EtOH.

5) Prior to use, make larvae food paste by combining with enough dH20 to form an applesauce-like consistency in a separate container.

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