Tracking Microbial Communities Across Aedes Albopictus Life Stages and Larval Habitat Types

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Title: TRACKING MICROBIAL COMMUNITIES ACROSS *AEDES ALBOPICTUS* LIFE STAGES AND LARVAL HABITAT TYPES

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Abstract

Aedes albopictus, the Tiger Mosquito, has been hailed as one of the most invasive arbovirus-transmitting mosquitoes globally. With the growing potential of microbial methods for mosquito control, it has become increasingly imperative to understand the factors that contribute to naturally-occurring microbiome communities. Here, we analyzed the impact of larval water type and life stage on the microbial community of *Aedes albopictus.* The field-collected water samples from tree holes and tires that were used to rear larval mosquitoes in the laboratory were significantly different from each other in terms of sOTU (bacterial species) richness, with tree holes having a far greater number of sOTUs. For beta diversity measures (Bray-Curtis dissimilarity) *Aedes albopictus* were not significantly different from adult *Aedes aegypti*, but mosquito sex, life stage, and overall treatment group were significantly different when analyzed by ANOSIM. Based on our findings, the environment surrounding larval mosquitoes (and subsequent adult mosquitoes from those habitats) and the life stage of mosquitoes (regardless of species) shapes mosquito microbiome assemblages. This work further supports the idea that

mosquito adults maintain a microbiome specific to larval habitat, despite major reductions to their microbiome prior to eclosion, which could shape the success of implemented microbial engineering or control methods.

Introduction

Aedes albopictus, or the Asian Tiger Mosquito, is an invasive container-breeding mosquito that has spread internationally from northern Asia over the past several decades and was likely introduced to North America beginning in the 1970's through the tire trade [1–4]. *Ae. albopictus* is rapidly spreading through the eastern United States, with the majority of the eastern US predicted to be occupied by 2080 [5,6]. The invasion of *Ae. albopictus* is particularly worrisome due to the arboviral transmission potential of this species. *Ae. albopictus* is a competent vector of Zika virus, Chikungunya virus, Japanese encephalitis virus, and dengue virus [4,7–10].

Recent work has analyzed the ability of environmental factors and the mosquito microbiome to alter the spread of arboviruses [11–16]. Mosquito microbiomes, as well as microbial methods of control like the introduction of *Wolbachia app.,* have the potential to inhibit arboviral establishment and thus reduce transmission in *Aedes* mosquitoes [15,17–21]. While several studies have been undertaken to understand microbiome-host-environment interactions in *Anopheles* mosquitoes, only a few studies have shown how microbiomes in *Aedes aegypti* are shaped by the environment [14,22–27]. In prior work in *Ae. aegypti,* microbial compositions differed by life stage and localized environments [14,23,26,28]. While it has become increasingly clear that environmental factors play a role in the formation of mosquito microbiomes, what remains unclear is how and which specific factors have the greatest influence in the formation of these microbial communities [11,13,14].

While we have some understanding of the microbiome-host-environment dynamics in *Ae. aegypti,* we know very little about how environmental factors may influence *Aedes albopictus* microbiomes and their vector competence. Here, we present a study analyzing the microbiome of *Ae. albopictus* under varying larval habitats and examining these impacts on microbiome over

54 life stages. We hypothesized, based on previous work in the closely related *Ae. aegypti*, that
55 *Aedes albopictus* microbial composition would differ across life stages and by larval habitat,
56 particularly between those that are naturally occurring, such as tree holes, and those that are
57 more anthropogenically derived, like tires.
58

Results

Microbiome Diversity

Alpha Diversity of the Microbiome: sOTU Richness

Kruskal-Wallis tests were run to examine differences in sOTU richness between all life stage experimental groups for *Ae. albopictus* and adults of *Ae. aegypti*. As is evident in Figure 1, there was no statistical difference between species (*Aedes albopictus* and *Aedes aegypti)* (p=0.585), days to eclosion (p=0.698), sex (p=0.119), life stage (p=0.563), or overall mosquito treatment group (p=0.134). Notably, there was a significant difference between the types of water used in the habitats during the experiment (p=0.049), with greater richness in the water samples collected from trees (*Fig. 2*).

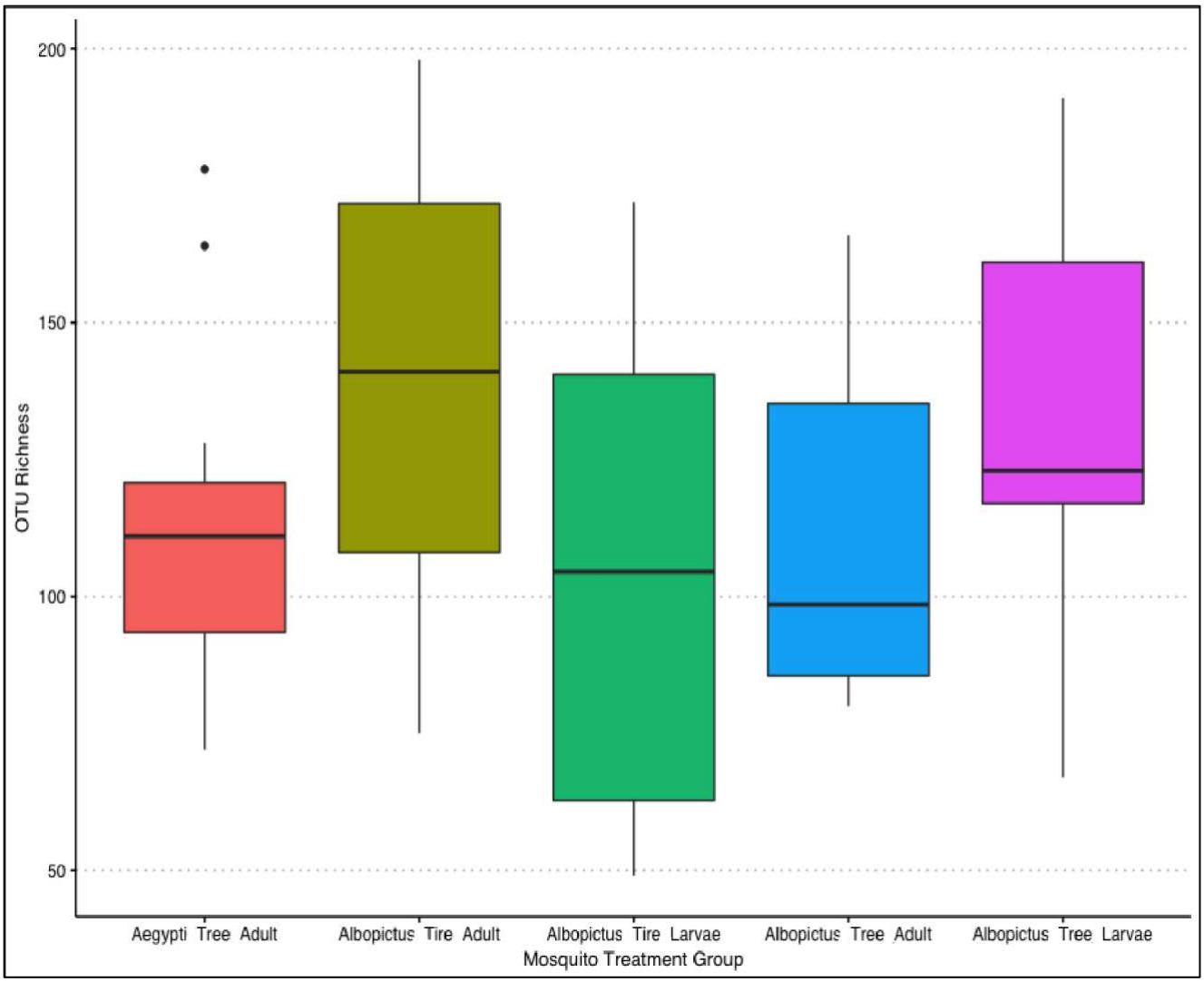

Figure 1. sOTU richness of the mosquito treatment groups across species, water habitat types, and life stage. There was no significant difference between life stage or water treatment groups when tested with a Kruskal-Wallis test (p=0.134). Sequences were rarefied to 2500.

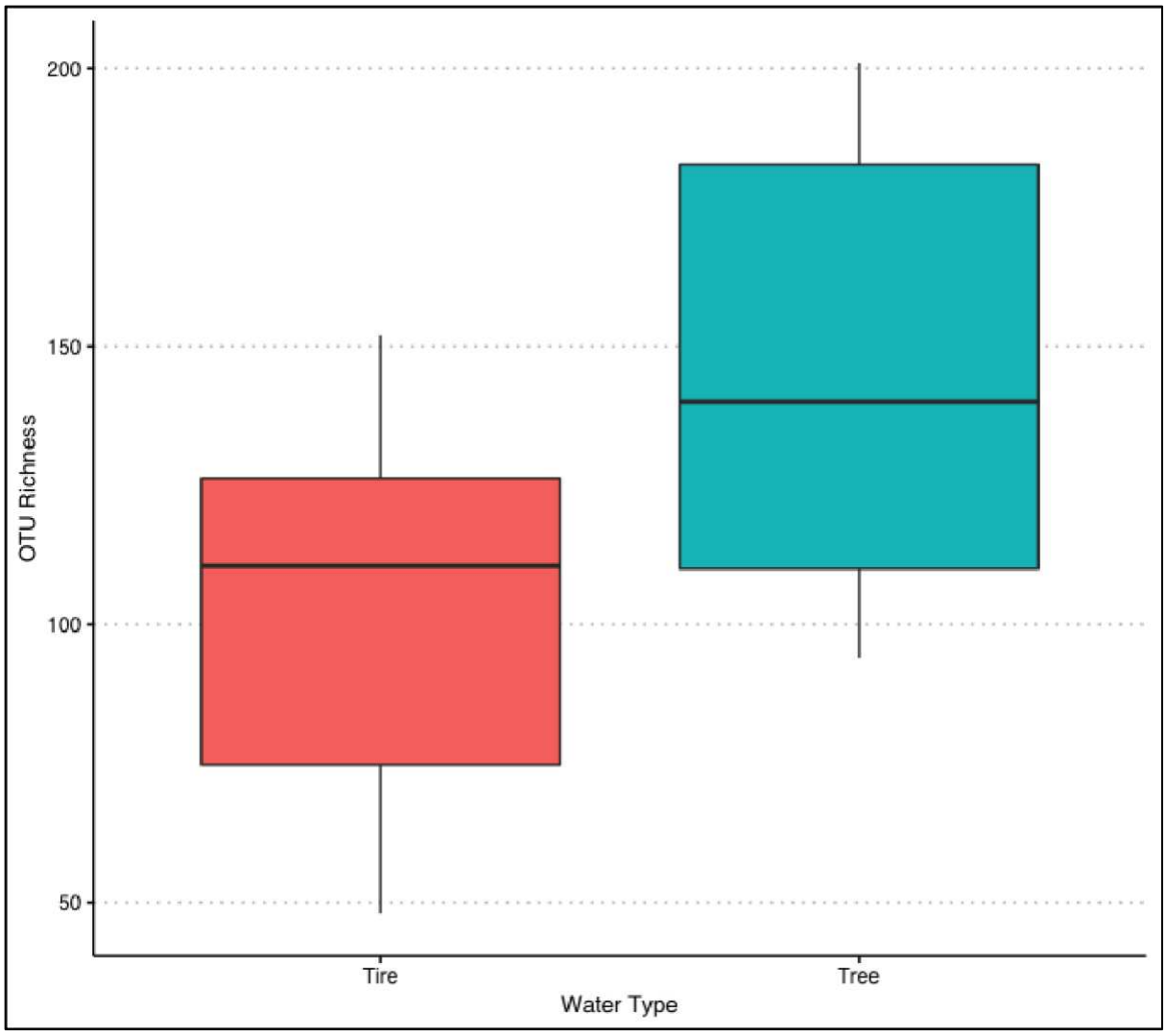

Figure 2. sOTU richness of water field-collected from two types of *Aedes* breeding sites, tree holes and tires, that were subsequently used to rear lab-derived mosquitoes. Tree and tire water richness was significantly different (p=0.049) by Kruskal-Wallis test, with greater sOTU richness in the tree samples.

Beta Diversity of the Microbiome: *Bray-Curtis Dissimilarity Index Distance Metrics*

Beta diversity was measured using Bray-Curtis dissimilarity index distance matrices analyzed by ANOSIM. Mosquito species were not significantly different (p=0.512), but adult mosquito sex (p=0.026), mosquito life stage (p=0.001; *Fig. 4*), and overall mosquito treatment group (p=0.001; *Fig. 3*) were significantly different. The sampling site of field-collected water from *Aedes* mosquito breeding sites- tree holes or tires- was also significantly different (p=0.001; *Fig. 5*).

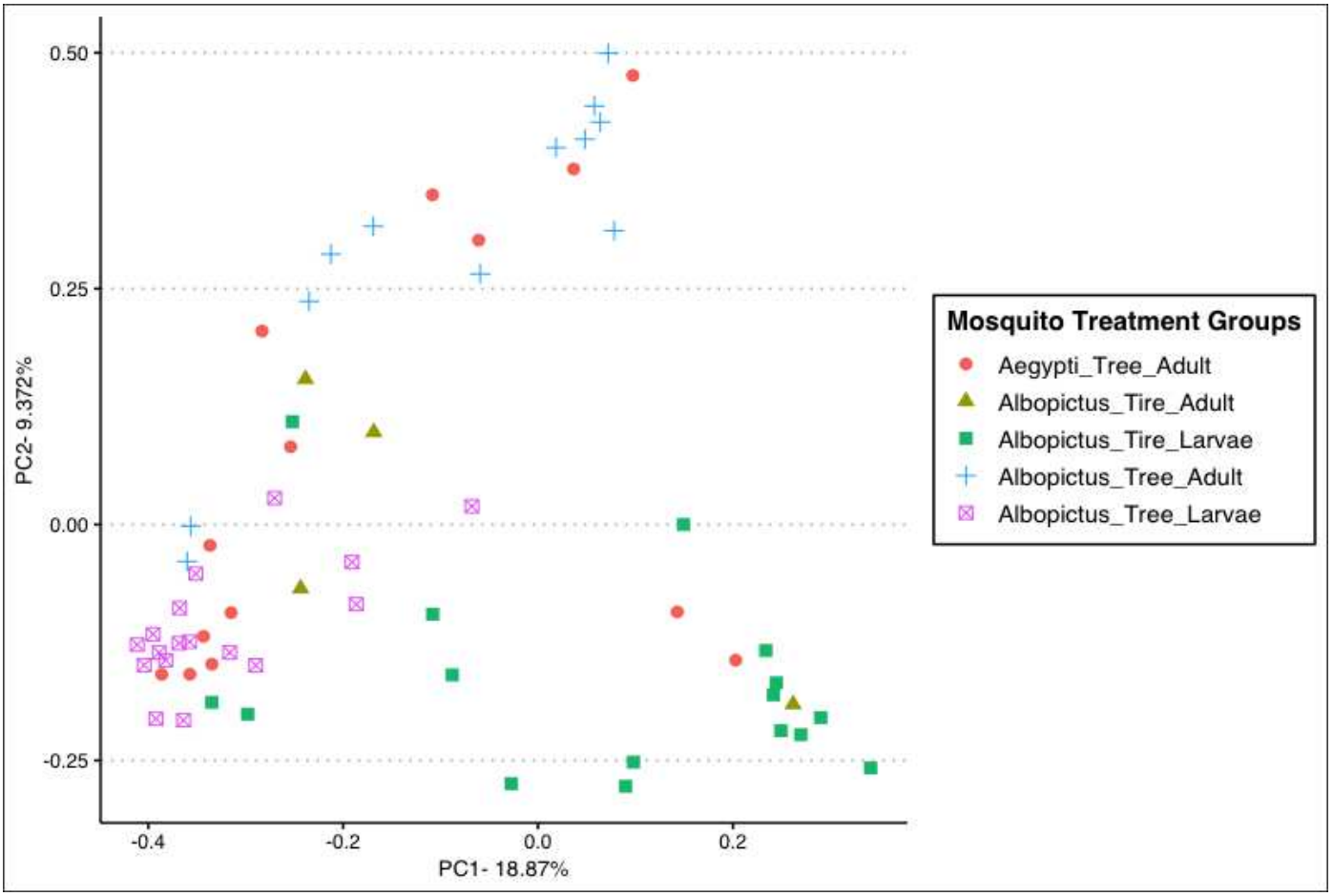

Figure 3. Principle coordinates analysis of overall mosquito treatment groups using Bray-Curtis dissimilarity index distances. Red circles represent *Aedes aegypti* adults reared in tree hole water

as a reference population, and the remaining sample groups are all *Aedes albopictus* mosquitoes. Yellow triangles represent *Aedes albopictus* adults reared in tire water, green squares are larvae reared in tire water, blue pluses are adults reared in tree hole water, and pink checked-boxes are larvae reared in tree hole water. *Aedes aegypti* adult samples cluster closely with *Aedes albopictus* adults from the same water source- tree holes. While the samples still diverged greatly by individual group, there was some overlap between samples of the same water type and samples of the same life stage.

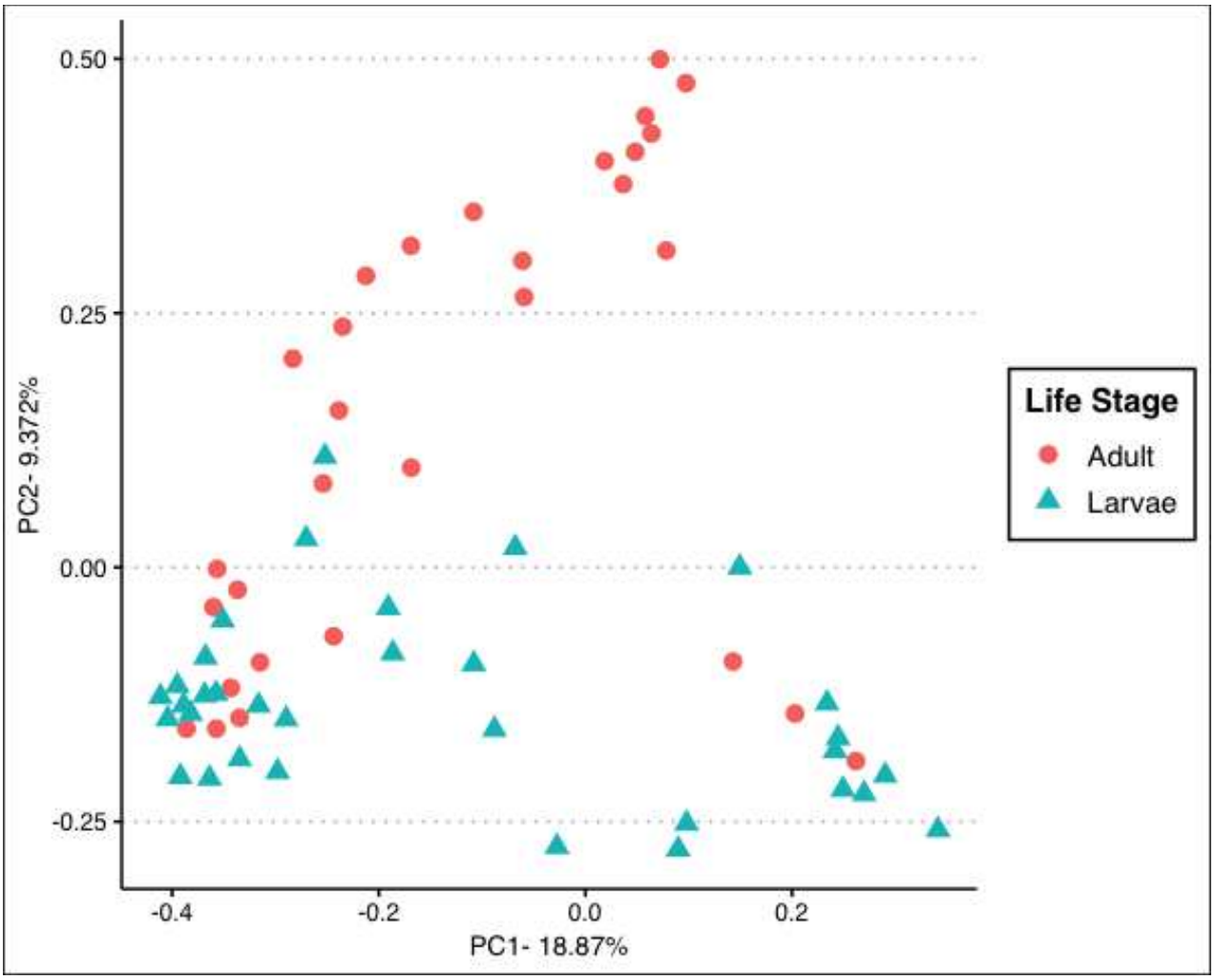

Figure 4. Principle coordinates analysis calculated using Bray-Curtis distances to analyze differences in microbial communities of mosquitoes at the adult and larval stages. Adult and larval microbiome assemblages diverge significantly (ANOSIM; p=0.001), but do retain some overlap.

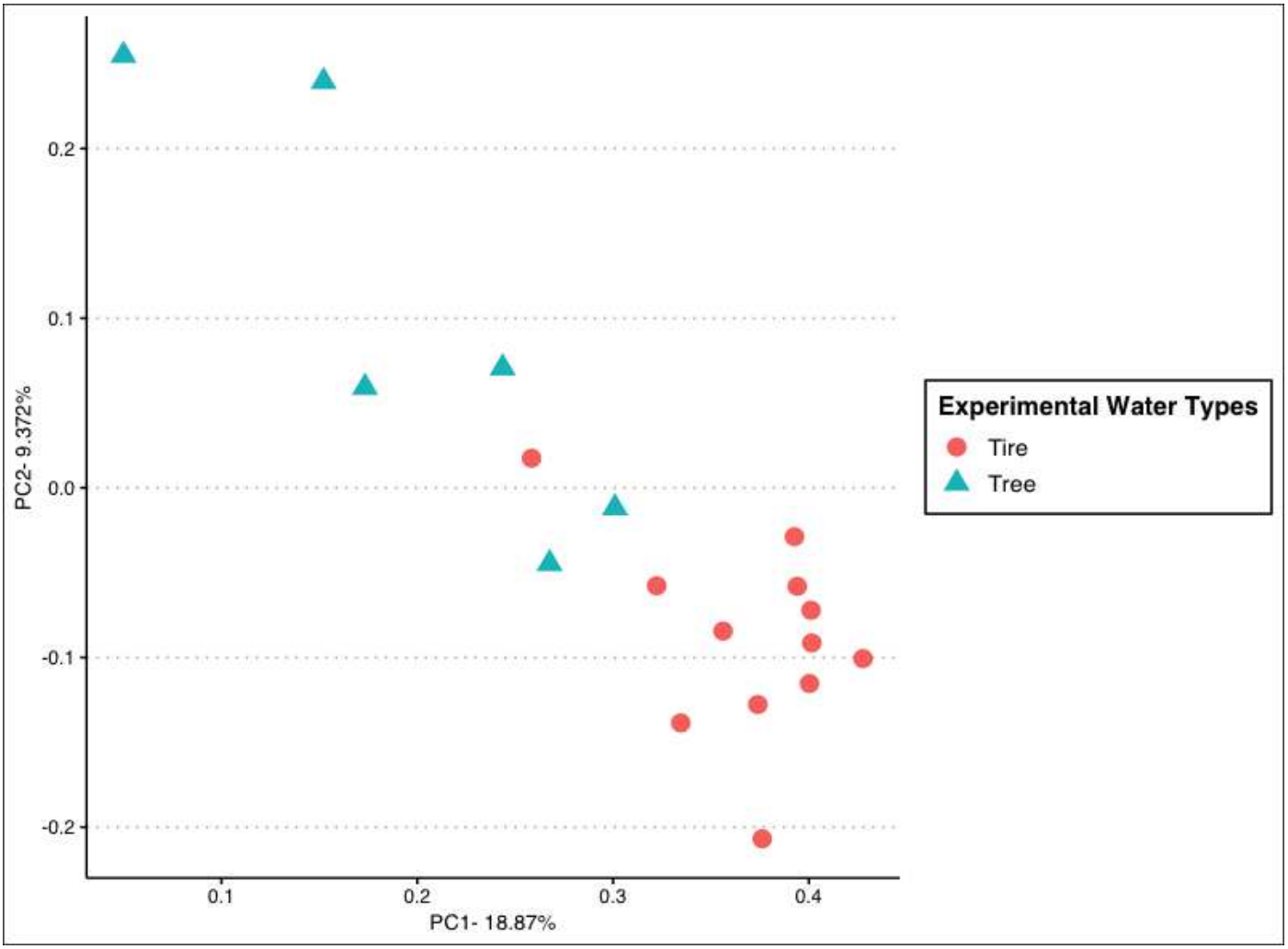

Figure 5. Experimental field-collected water types from mosquito breeding sites in a principle coordinates analysis. Tire and tree hole samples differ greatly in their microbial communities' composition (ANOSIM; p=0.001), though there is some minimal intersection.

Average Taxonomy of the Mosquito Microbiome

Mosquitoes and the water from their larval habitats were similar in microbial membership (Kruskal-Wallis, p=0.157), though the proportions of these microbes are significantly different (ANOSIM, p=0.001) (*Fig. 6*). The most common bacteria across all samples were in the families *Comamonadaceae*, *Rhodocyclasceae, Oxalobacteraceae, Sphingomonadaceae,* and *Moraxellaceae.* While there were differences in many of the taxa, we will highlight a few groups

of interest from the average taxa by experimental group. The tree water type was much more diverse in terms of both alpha and beta diversity, with noteably more groups of sOTUs present than the tire-derived water samples. Interestingly, *Rhodocyclaceae* and *Moraxellaceae* were found in very small relative abundances in all water samples but were much higher abundance in mosquito larvae and adults, regardless of species or water type. The inverse pattern was seen in *Oxalobacteraceae*.

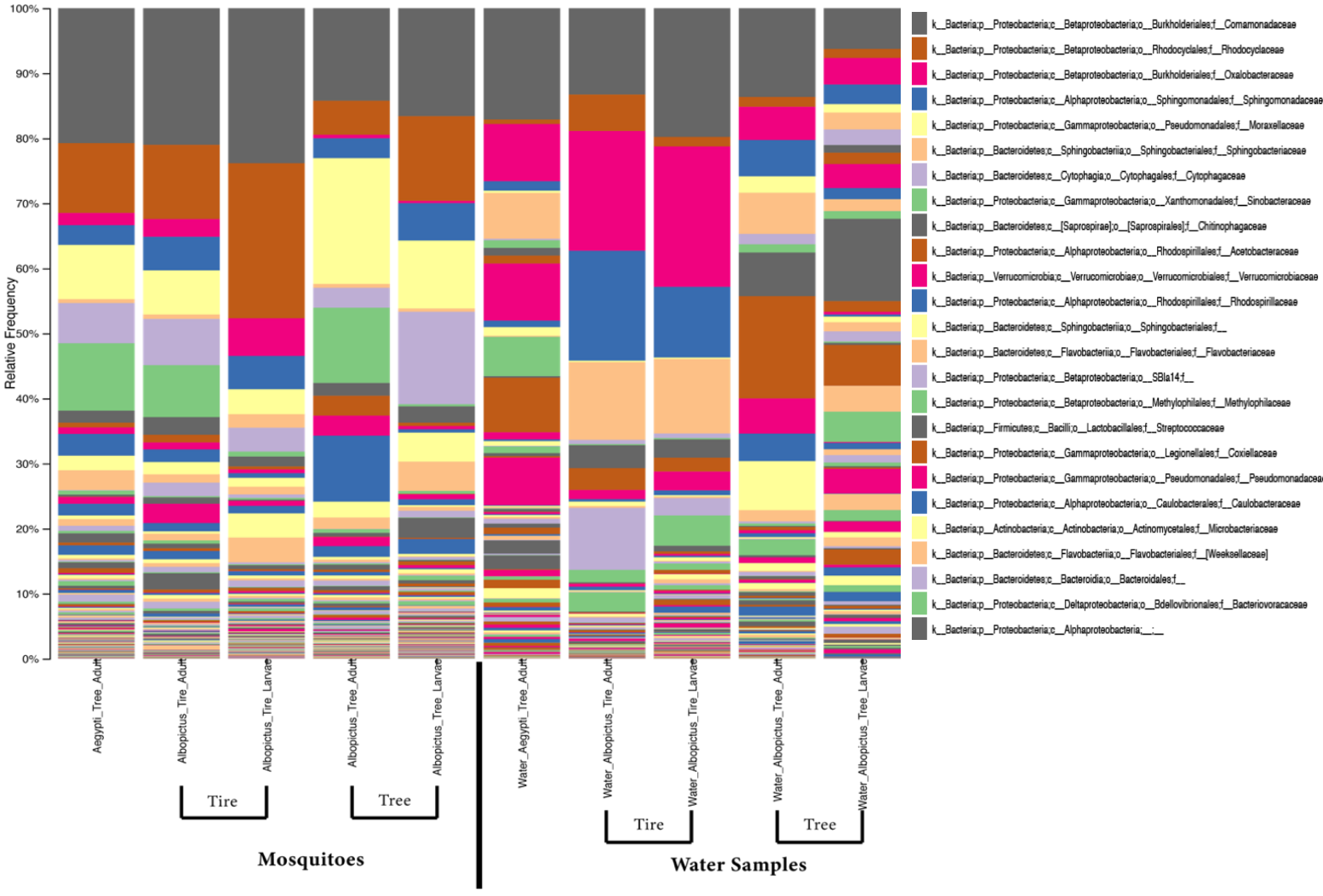

Figure 6. Average family-level taxa present in all mosquito and water samples. While there is substantial overlap in the members of the average microbiome of each experimental group, differences between the proportions of those microbes are prominent. Most evidently, the

relative abundances of bacteria in the water samples differ greatly between tire and tree hole water (i.e. *Oxalobacteraceae, Sphingomonadaceae,* and *Moraxellaceae*), and the water samples overall differ considerably from the mosquito samples (*Rhodocyclaceae, Oxalobacteraceae,* and *Moraxellaceae*). The reduced legend pictured here includes the families of the 25 most abundant sOTUs across all samples, and the expanded legend can be found in Supplemental Figure S4.

Mosquito larvae and adults derived from a common water source had very similar microbiome compositions (i.e. tree hole water larvae looked like tree hole adults). The relative abundances between mosquitoes raised in tree or tire water differed substantially (ANOSIM, p= 0.001), though they had most of the sOTUs in common and closely reflected the water habitat in which they were reared. Interestingly, *Sinobacteraceae* were minimally present in the larvae reared in either water type but were prevalent in the adult mosquitoes.

Discussion

As the field of mosquito microbiome studies has expanded in recent years, questions regarding the initial formation of these microbiomes have persisted. One major question that has endured is the relative roles of environment, host phylogeny, or host life stage in the formation of the microbiome. Here, we characterized the microbiome of *Aedes albopictus* reared in field-collected water samples and determined several drivers of microbial establishment in these mosquitoes.

Microbial Diversity

In the analysis of the sOTU richness of mosquito samples, there were no differences across any experimental groups, including species, sex, or life stage, but there was a significant difference between the richness of water samples from trees and tires. This indicated that the environment that mosquitoes were exposed to were significantly different from each other in terms of alpha diversity. The beta diversity of the water types was also different, further differentiating the starting environments that mosquito larvae were subjected to. However, the number of microbes present in mosquitoes was consistent across all treatments, indicating that both larval and adult mosquitoes can control for the number of microbes able to colonize them, essentially filtering the environmental microbes.

There were significant differences in the community structure between mosquito sexes, life stages, and the water types sampled, but there was no difference between *Aedes albopictus* and the reference population of *Aedes aegypti.* The average taxa analysis of the microbiome by each experimental group showed that these samples were extremely similar in composition, and very closely reflected the assemblage of their water, despite their species. These analyses clearly

indicate that for closely related mosquito species, microbiome assemblage depends more on environmental factors than on genetic background.

Additionally, there was a clear difference in the proportions of several major microbial taxa (and the overall beta diversity) between mosquitoes reared in tire or tree hole water. These proportions of microbes mirrored the water type that mosquitoes were raised in, despite the mosquitoes acting as a microbial filter. This further supports the larval aquatic environment and its microbes driving the formation of the mosquito microbiome.

There was also a significant difference in beta diversity between mosquito life stages. While both adult and larval mosquitoes looked like a proportionally-altered version of their water environment, the difference in life stages supports the hypothesis that mosquito hosts have a limited control over their microbial assemblage. As mosquitoes' immune system function differs at the larval and adult stages, what influence mosquitoes do have to filter their microbiome is altered at each stage [19,29–32].

Findings and Impacts

Our findings indicate that mosquito microbiomes closely reflect their larval habitat with some modulation by the mosquito host. We conclude that mosquitoes, particularly *Aedes albopictus,* are colonized by microbes from their larval environment, lose a substantial proportion of their microbiome as pupae through eclosion [33], and then are recolonized by the microbes specific to that location after eclosion (*Fig.* 7). Alterations in the adult mosquito gut microbiome and reduction in microbial diversity with nectar or blood feeding are also well documented [23]. While the mechanism of this re-colonization after eclosion is unclear, we propose the period of mosquito resting on the surface of the water post-eclosion as a potential point for

reseeding of the microbiome, similar to the partial colonization of humans by microbes from the home environment [34–36].

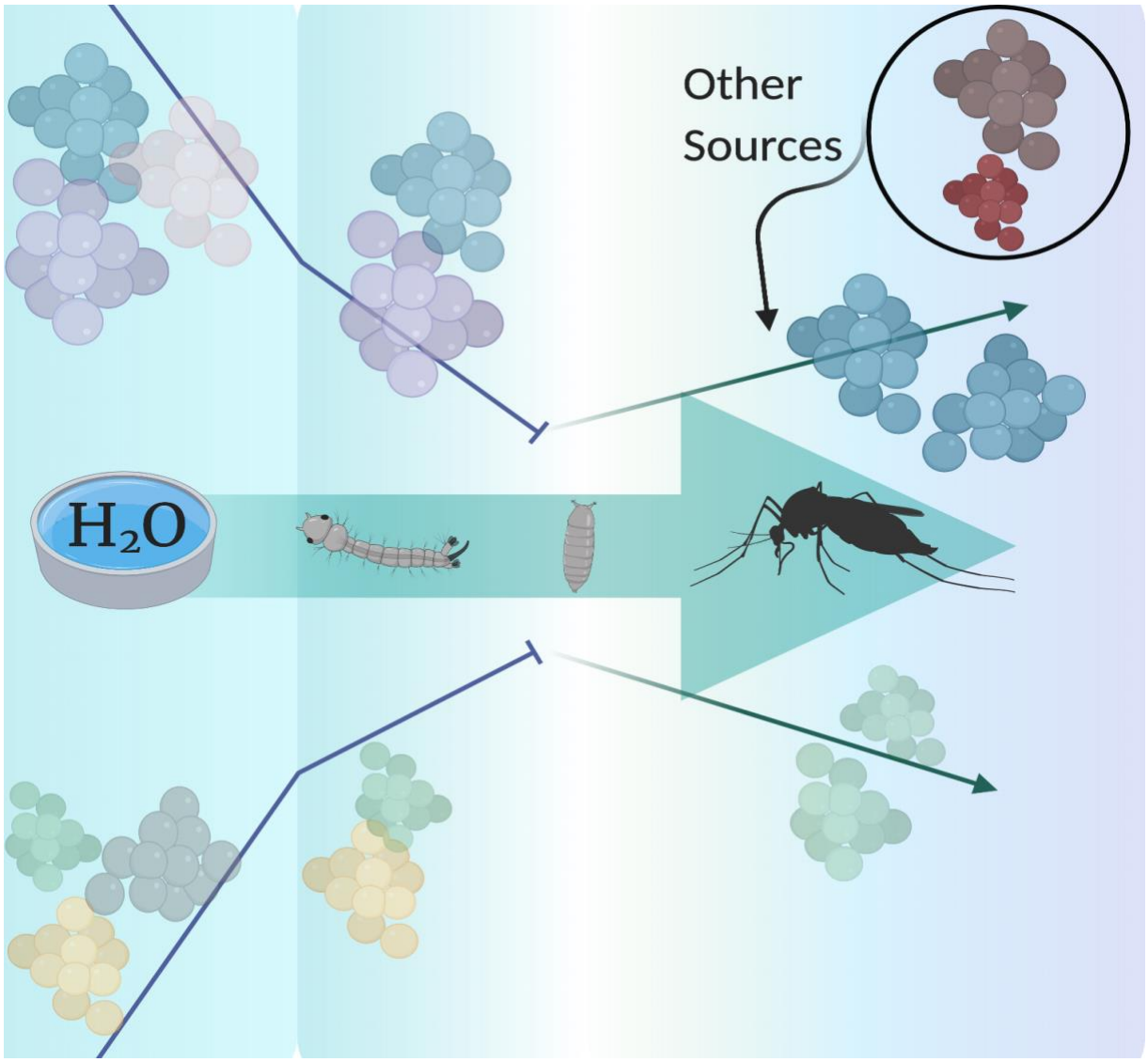

Figure 7. Mosquitoes closely reflect, but also act as a filter, for the microbial community of their larval habitat. When mosquitoes prepare to eclose, they lose much of their microbiome but regain it shortly after eclosion. Adults reflect their larval environment incompletely but with specificity, and with the addition of new members of the microbiome present in the adult that were not present in the larval habitat and are derived from other terrestrial sources. The microbiome further shifts with the introduction of meal sources, whether nectar or blood.

Our conclusions are further supported by prior work on microbiomes in the laboratory, mesocosms, and laboratory-field combination experiments in *Aedes* mosquitoes [13,14,16,23,26,37]. Coon et al. (2016) found that field-collected and lab-reared mosquitoes required microbial symbionts for successful growth and also revealed site specificity in mosquito microbiomes at closely located field sampling sites. Dickson et al. (2017) found differences in the microbial communities of mosquito breeding sites and that these led to incongruences in anti-bacterial and anti-dengue virus activity by larval habitat. These prior findings along with our novel investigation indicate that microhabitat is an important factor in mosquito larval microbiome formation and has life-long impacts on adult mosquitoes and their microbial communities [19,38].

Coon et al. (2016) indicates that mosquito larvae require bacteria to survive and thrive, but that *Aedes* larvae are most likely generalists in terms of their reliance on microbes with relatively low host-microbe specificity. Bennett et al. (2019) found low intra-specific variance between *Aedes aegypti* and *Aedes albopictus* microbiome diversity, and that *Aedes* mosquito microbiome assemblages could not be easily explained by geographic variables, container types, or specific elements of the aquatic environment alone. While our novel results provide further evidence regarding the importance of microhabitat in the formation of mosquito microbiomes, these findings taken together indicate that mosquito microbiomes are driven by a complex combination of environmental factors that require further elucidation. Building on our understanding of how mosquito microbiomes are formed and which environmental parameters are most important in their formation will help to guide the implementation of microbial methods of mosquito and arboviral control.

Methods

Site Selection and Water Collection

Water collection sites for use in the experiment were determined by the presence of known breeding populations of *Aedes albopictus* in New Bedford, Massachusetts, USA. Two cemeteries with many tree holes filled with water and an abandoned lot with abundant debris, including containers and tires filled with standing water suitable for mosquito habitat, were sampled from this area. Tree holes and tires, which represent natural and anthropogenic breeding containers for *Aedes* mosquitoes, were sampled by using a sterile transfer pipette to remove 50mL of water found in these locations to a sterile conical tube for each tree or tire. Water samples were transported in a cooler to minimize alterations to the water microbiome from field conditions and the experiment was set up in the same day that the water samples were collected. Ten samples from primarily beech trees and ten samples from degraded, undisturbed tires were sampled. Water samples were sub-sampled and then combined by overall water type to create a composite water composition and limit microhabitat variance between individual tires or trees. These composite water types of tire or tree hole were then passed through a sterilized large-pore sieve to remove large debris and any macropredators before further use in experiments.

Laboratory Mosquito Husbandry

In order to keep genetic background between mosquitoes similar and to reduce the likelihood of local adaptations of *Aedes albopictus* to our field-collected water sources, mosquitoes were lab-reared and from stocks outside of the American Northeast. *Aedes albopictus* eggs were third generation lab-reared mosquitoes derived from field-collected mosquitoes in southern Texas. *Aedes aegypti* (Gainesville strain) eggs were ordered from

Benzon Research (Carlisle, Pennsylvania, USA). *Aedes aegypti* were used as a reference population since prior work on these exists. All mosquito larvae were hatched concurrently under standard incubator conditions in a Percival I-36VL incubator (Perry, Iowa, USA) in a sterile cage and containers at 21°C and 65% humidity, with a 14:10 light:dark cycle, and in sterile pond water (sterile molecular grade water re-ionized with salt concentrations equivalent to that of a pond and autoclaved). Mosquito larvae were not fed until the start of their water exposure at the very start of their second instar.

Experimental Design

Individual second instar mosquito larvae were placed randomly into 5mL of either composite tree hole or tire water in a larger 15mL sterile conical tube. Extra headspace was maintained in the tubes to reduce ambient transfer of microbial materials and to minimize any potential of water splashing. Each conical tube was then covered with a sterilized 4x4 cm square of mosquito mesh and a sterilized fabric-coated elastic. Mosquito tubes were randomly placed in tube racks and kept in the cleaned biosafety cabinet (Labconco, 6 foot, A1 Class 2, Kansas City, MO, USA) at 21°C, approximately 35% humidity, and a 14:10 light cycle. Tube racks were rotated daily to avoid biases in light availability in the biosafety cabinet. Tubes were checked 2-3 times daily for development, eclosion, and mortality events.

As mosquitoes reached fourth instar, 20 randomly selected larvae of each experimental group (species + water type) were removed from the experiment, euthanized by freezing, and had 500uL of pre-chilled RNALater ICE (ThermoFisher Scientific, Waltham, MA, USA) added to them for safe freezer storage at -20°C. The remaining mosquitoes were then allowed to continue their development through approximately 12 hours post-eclosion. Mosquitoes were then

273 euthanized by freezing and had RNALater ICE added. In total, 58 adults and 40 larvae were
274 sampled, in addition to the waters that they developed in.
275
276 **Specimen Storage and Extraction**
277 Specimens were stored in the -20°C freezer in RNALater ICE and were kept frozen until
278 one large batch extraction could be performed at the end of the experiment to minimize
279 extraction batch biases. Extractions were performed using the Quick-DNA/RNA MagBead
280 extraction kit (Zymo Research, Irvine, California, USA). All mosquito samples were bead-beaten
281 at maximum vortex-adaptor speed for 15 minutes using ZR BashingBead Lysis Tubes (0.1 &
282 0.5mm; Zymo Research) with 750uL of DNA RNA Shield (Zymo Research) added to the tubes
283 to preserve the samples. Lysis buffer and Proteinase K were then added to the bead bashing
284 tubes, mixed well, and left at room temperature for a 90 min incubation. Effluent was then
285 transferred to a 96-well deep plate for the remainder of the Zymo "DNA and RNA Purification"
286 extraction protocol option.
287 Water samples (~5mL) were filtered onto a sterile 0.22um filter, were capped, and filled
288 with lysis buffer and proteinase K and left to incubate. After 90 min, the cap was removed and
289 the effluent was pushed with a sterile syringe into a new sterile tube for transfer to the 96-well
290 plate for the rest of the extraction protocol. Water and mosquito samples were randomized across
291 the 96-well plates to minimize plate, edge, or grouping effects.
292
293 **Microbiome Sequencing and Preparation**
294 PCR was performed to amplify the 16S rRNA V4 gene region using the Earth
295 Microbiome Project standard 515F and 806R primers (barcodes on the forward primer) and

protocols [39–43]. Samples and controls were amplified in duplicate using 5Prime HotMasterMix (Quantabio, Beverly, Massachusetts, USA) (10ul), Milli-Q Ultrapure Water (8.5ul), 10uM 806R (3.5uL), 10uM barcoded 515F (1uL), and template DNA (2uL) for a total of 25ul per well. Cycling conditions followed the standard EMP protocol with minor adjustments for the Quantabio 5Prime HotMasterMix and can be found in Table 1.

Temperature (°*C*)	**Time**	**Number of Cycles**
94	3 min.	1x
94	45 sec.	35x
50	60 sec.	
72	90 sec.	
72	10 min.	1x
4	Continuous	Continuous Until Removed

Table 1. PCR Conditions for the amplification of the 16S rRNA V4 region gene using the barcoded-515F and 806R Earth Microbiome Project primers.

The Mag-Bind Pure Library Normalization Kit (Omega Bio-Tek, Norcross, Georgia, USA) was used to normalize PCR-amplified samples. Sampled were pooled for library quantification and subsequent dilution using a Qubit 2 Fluorometer (Invitrogen, Carlsbad, California, USA). PhiX was added according to recommendations from the Earth Microbiome Project and the library was sequenced using an Illumina MiSeq v2 300-cycle kit [44,45].

Bioinformatic and Statistical Analyses

Analysis of mosquito microbiomes was performed using QIIME2 and R, with R packages vegan, microbeR, and qiime2r [46–50]. Raw sequences were demultiplexed and deblurred in QIIME2, and were rarefied at 2500 sequences [39,51,52]. Alpha (sOTU Richness) and beta (Bray-Curtis dissimilarity index distances) diversity metrics were statistically examined between experimental groups using Kruskal-Wallis and ANOSIM tests in QIIME2, respectively. Average taxa for each experimental group were generated in QIIME2 by grouping and collapsing sOTUs within each group and averaging the sOTUs present in the average sample from that group.

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Author Contributions

ATP and DCW conceptualized and designed the study. ATP executed sample collection, performed all experiments, and analyzed resulting data. ATP wrote the manuscript and DCW provided scientific direction on the manuscript and project. ATP and DCW both sourced funding for the study and reviewed all portions of the manuscript.

Competing Interests

The author(s) declare no competing interests.

Data Availability

The datasets generated and analyzed during the current study are available from the corresponding author on reasonable request.

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Figure Legends

Figure 1. sOTU richness of the mosquito treatment groups across species, water habitat types, and life stage. There was no significant difference between life stage or water treatment groups when tested with a Kruskal-Wallis test (p=0.134). Sequences were rarefied to 2500.

Figure 2. sOTU richness of water field-collected from two types of *Aedes* breeding sites, tree holes and tires, that were subsequently used to rear lab-derived mosquitoes. Tree and tire water richness was significantly different (p=0.049) by Kruskal-Wallis test, with greater sOTU richness in the tree samples.

Figure 3. Principle coordinates analysis of overall mosquito treatment groups using Bray-Curtis dissimilarity index distances. Red circles represent *Aedes aegypti* adults reared in tree hole water as a reference population, and the remaining sample groups are all *Aedes albopictus* mosquitoes. Yellow triangles represent *Aedes albopictus* adults reared in tire water, green squares are larvae reared in tire water, blue pluses are adults reared in tree hole water, and pink checked-boxes are larvae reared in tree hole water. *Aedes aegypti* adult samples cluster closely with *Aedes albopictus* adults from the same water source- tree holes. While the samples still diverged greatly by individual group, there was some overlap between samples of the same water type and samples of the same life stage.

Figure 4. Principle coordinates analysis calculated using Bray-Curtis distances to analyze differences in microbial communities of mosquitoes at the adult and larval stages. Adult and larval microbiome assemblages diverge significantly (ANOSIM; p=0.001), but do retain some overlap.

Figure 5. Experimental field-collected water types from mosquito breeding sites in a principle coordinates analysis. Tire and tree hole samples differ greatly in their microbial communities' composition (ANOSIM; p=0.001), though there is some minimal intersection.

Figure 6. Average family-level taxa present in all mosquito and water samples. While there is substantial overlap in the members of the average microbiome of each experimental group, differences between the proportions of those microbes are prominent. Most evidently, the relative abundances of bacteria in the water samples differ greatly between tire and tree hole water (i.e. *Oxalobacteraceae, Sphingomonadaceae,* and *Moraxellaceae*), and the water samples overall differ considerably from the mosquito samples (*Rhodocyclaceae, Oxalobacteraceae,* and *Moraxellaceae*). The reduced legend pictured here includes the families of the 25 most abundant sOTUs across all samples, and the expanded legend can be found in Supplemental Figure S4.

Figure 7. Mosquitoes closely reflect, but also act as a filter, for the microbial community of their larval habitat. When mosquitoes prepare to eclose, they lose much of their microbiome but regain it shortly after eclosion. Adults reflect their larval environment incompletely but with specificity, and with the addition of new members of the microbiome present in the adult that were not present in the larval habitat and are derived from other terrestrial sources. The microbiome further shifts with the introduction of meal sources, whether nectar or blood.

Supplementary Files

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