INVESTIGATING PATTERNS AND VARIATIONS IN *AEDES ALBOPICTUS* (DIPTERA: CULICIDAE) AND ITS MICROBIOME IN HAWAII

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Dedication

This work is dedicated to the memory of my sister Valerie Kenerson, and my grandmother Ernestina Sandoval, whom both passed away before I was able to finish my Ph.D. To my sister Valerie, thank you for encouraging me throughout the years, and loving me through your pain. I only wish you could be here to see how far I have come. A mi abuela, gracias por estar siempre ahí para mí, y animarme a terminar la escuela. Te extraño mucho, y ojalá pudieras verme finalmente terminar mi carrera.

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ABSTRACT

Understanding the drivers of distribution and abundance of invasive species remains a significant challenge especially on the Hawaiian Islands. Mosquitoes (Diptera: Culicidae) are non-native to Hawai'i and the diseases they vector are a contemporary threat to human and wildlife health. Despite being a well-established mosquito species, *Aedes albopictus* (Skuse) distribution and the factors influencing the population and its host associated microbiome in Hawaiʻi are poorly understood. In this study, I investigate how the environment influences the distribution and abundance of *Ae. albopictus* and the diversity and distribution of its host-associated microbiome in Hawaiʻi.

In a yearlong island-wide surveillance study on Maui, the patterns of mosquito distribution were evaluated in relation to environmental factors (rainfall and temperature), elevation, space, and time using both oviposition and adult traps. Overall, four mosquito species were detected, *Ae. albopictus*, *Ae. vexans*, *Culex quinquefasciatus*, and *Wyeomyia mitchellii*. The survey determined that *Aedes albopictus* is abundant and widely distributed around Maui, and adult abundance and oviposition varied with site and time of collection. The oviposition of *Ae. albopictus* was positively associated with sites that are warm and low elevation, while the abundance of adults was not influenced by the environmental variables. The complex characteristics of each site such as vegetation, land use and host availability likely influenced the abundance of adult *Ae albopictus* and its ovipositional activity. However, *Culex quinquefasciatus*, *Ae. vexans*, and W. *mitchellii* were not frequently collected, and were not assessed in relation to space, time, or environmental variables. The trapping methods and frequency and intensity of sampling are likely to have been the main drivers behind the probability that these species and others were collected. Nonetheless, these findings can be used provide a baseline for future mosquito surveillance studies to assess new mosquito species

incursions, and range expansion.

Adult *Ae. albopictus* collected in the above survey were used to assess host associated microbiome in relation to space, time, rainfall, temperature, and host infection with a co-occurring pathosymbiont *Ascogregarina taiwanensis*. The microbiome of *Ae. albopictus* was found to vary across the island and be influenced by infection status with *A. taiwanensis*. The microbiome of *Ae. albopictus* did not shift in accordance with gradients of temperature or rainfall, suggesting that mosquitoes actively maintain their microbiome. As with the distribution of the adult mosquitoes, the complex factors associated with various sites, are diving the differences in the host-associated microbiome across geographic location. This study also highlights that inter- and intra-domain interactions may structure the *Ae. albopictus* microbiome. Cumulatively, understanding the explicit drivers of the mosquito microbiome assembly may have important implications in developing mosquito control strategies.

I further explored how the microbiome is heterogeneous within the host and demonstrate that the environment provides the source of microbes. Results indicate that the microbiome of *Ae. albopictus* tissues is compositionally nested within the environmental microbiome i.e. microbes within the mosquito tissues are also found in the soil, water and on plants in the mosquito habitat. Within the mosquito, the microbiomes of the crop, midgut, Malpighian tubules, and ovaries differed in composition. The microbiomes of mosquitoes tissues formed two specialized modules, one in the crop and midgut and another in the Malpighian tubules and ovaries. The specialized modules may form based on microbe niche preferences and/or selection of mosquito tissues on specific microbes that may aid unique biological functions of the tissue types. Strong niche-driven assembly of tissue-specific microbiotas from the environmental species pool suggests that each tissue has specialized associations with microbes, which perhaps derive from host-mediated microbe selection.

Overall, this study investigated the drivers of distribution, and abundance of *Ae. albopictus* and additionally the diversity, distribution and abundance of the host-associated microbiome present in Hawaii. These findings fill an important gap in understanding about a significant disease vector and provide information for future surveillance and studies, particularly those that lead to the development of novel methods of mosquito management.

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LIST OF ABBREVIATIONS

Mosquito Borne Disease	MBD
West Nile Virus	WNV
Chikungunya	CHIK
Japanese encephalitis virus	JEV
La cross virus	LACV
Dengue virus	DENV
Zika virus	ZIKAV
Eastern equine encephalitis virus	EEEV
Rift Valley fever virus	RVFV
Yellow fever virus	YFV
Center for Disease Control	CDC
Biogents	BG
Clustered regularly interspaced short palindromic repeats	CRISPR
Haleakala National Park	HAK
Haleakala Road 1	HAK1
Haleakala Road 2	HAK2
Hana Hwy 1	HAN1
Hana Health	HANH
Kahului	KAH
Kahului Airport	KAH-AIRPT

Kaumahina State Park	KAUM
Kaupo	KAUP
Kealia Pond	KEA
Keokea	KEO
Kipahulu	KIP
Kula	KUL
Lahaina	LAH
Maui Forest Bird Recovery	MFBR
Maui Invasive Species Council	MISC
Olowalu	OLO
Waihee	WAI
Wakina	WAK
Aedes albopictus	AA
Culex quinquefasciatus	CQ
Aedes vexans	AV
Wyeomyia mitchellii	WM
Operational Taxonomic Unit	OTU

CHAPTER 1

INTRODUCTION

OVERVIEW

Mosquitoes (Diptera:Culicidae) capable of vectoring human and zoonotic pathogens are responsible for significant impacts to human and zoonotic health (1-3). Mosquito-borne diseases (MBDs) remain a contemporary public health problem with disparate impacts on low to middle income countries (4-6), and in particular on island nations (7). The increase of MBD is linked to the rapid spread of mosquitoes around the world, driven primarily by globalization (8), deforestation (8), and climate change (9). After decades of research, there are now a number of ways in which to interrupt the MBD cycle. The most effective approach has been to regulate vectors, which requires a thorough understanding of vector biology, breeding sites and reliable population monitoring and effective management tools (7, 10).

Aedes albopictus Skuse, is one of six species of mosquitoes that have been introduced into Hawaiʻi (11). A globally-distributed vector of arboviruses, *Ae albopictus* has expanded its distribution into Europe in the last 10 years (12), and is a vector of Dengue (DENV), Zika (ZIKAV) and Chikungunya viruses (CHIK) (13) among others. Despite being a well-established mosquito species in the Hawaiian Islands, *Ae. albopictus* distribution and the factors influencing the population in Hawaiʻi are poorly understood. The gap in knowledge of mosquito distribution in Hawai'i is largely due to budget cuts (14) that have led to restrictions in surveillance. Furthermore, vector surveillance in Hawaiʻi is focused on ports of entry to detect new mosquito species incursions (15). While surveillance at ports is extremely important, the island-wide distribution of mosquito populations remains poorly resolved. This has been partially addressed on Oʻahu and the island of Hawaiʻi where comprehensive distribution maps have been created and management plans have been created based on this understanding. However, at present, outer islands such as Maui, Molokaʻi, and Lānaʻi have not had extensive island-wide surveillance.

Furthermore, the biotic and abiotic factors influencing mosquito populations dynamics in Hawaiʻi are likewise poorly understood, specifically with *Ae. albopictus* which is widespread throughout the state, and globally a significant vector of human pathogens. Therefore, it is critical to monitor island-wide mosquito populations, by preparing comprehensive distribution maps and assessing how seasonality, and environmental gradients influence mosquito populations. Such knowledge will enhance vector control strategies, and further protect the community from mosquito-borne disease transmission.

Novel approaches to reducing mosquito-borne disease transmission involve using an understanding of the microbiome in local mosquito populations to produce management tactics (16-19). The mosquito microbiome alters the physiological traits of medically important mosquitoes by interfering with reproduction (20), steering the course of development (21, 22), and stimulating the immune system (3, 23), and these effects influence the ability of mosquitoes to sustain disease transmission (16, 19, 24-27). The microbiotas maintain diverse interactions with their host that vary from mutualisms (where both the microbe and host benefit) to parasitism's (where the host face a negative cost for its association with the microbe) (28). The composition of the mosquito microbiome varies significantly across mosquito populations. Furthermore, the factors that contribute to this variation are poorly understood (29, 30) and have not been studied in Hawaiian populations of *Ae. albopictus*. Investigating these factors will help to develop successful disease suppression strategies for this important disease vector

This study aims to identify drivers of variation in *Ae. albopictus* mosquito populations and its associated microbiome in Hawaiʻi. *Aedes albopictus* is widespread, and readily transmits several viruses. Conducting an island-wide survey will clarify the distribution and abundance of mosquitoes on the island of Maui, which is currently unknown, and will specifically demonstrate how widespread *Ae. albopictus* is on this island. This study will identify biotic and abiotic factors that influence *Ae. albopictus* distribution, abundance, and its host associated microbiome. Results from this study will fill in knowledge gaps and inform mosquito management strategies.

MOSQUITOES IN HAWAIʻI

Hawaiʻi is a volcanic archipelago in the Pacific Ocean with a high level of endemism and an invasive species hotspot (31). There are no species of mosquito recognized as being native to the Hawaiian Islands (11). The presence of mosquitoes in Hawaiʻi was first noted in 1826, when *Culex quinquefasciatus* Say arrived on cargo ships (11). Since then, there have been six species that have arrived in Hawaiʻi likely with cargo ships and air travel (11) (Table 1.1). *Aedes albopictus* was introduced in Hawaiʻi at the end of the 17^{th} century (32). *Aedes albopictus,* is a globally distributed vector of human pathogens, and is able to breed in natural habitats (tree holes, bamboo stumps, and bromeliads), and is also adapted to suburban and urban environments (32). The ability of *Ae. albopictus* to adapt to a variety of environments and has led its widespread distribution in the state of Hawaiʻi, although its exact distribution, specifically on outer islands such as Maui, Lānaʻi and Molokaʻi are unknown. There has been some distributional work on the island of Hawaiʻi, however the state-wide distribution of *Ae. albopictus* is unknown (33). In an attempt to control *Ae. albopictus* mosquitoes, mosquitoes of the *Toxorhynchites* genera were introduced into Hawai‘i as the immature stages of this mosquito feed on larvae found in the same aquatic habitat. In the time period from 1929-1950s, *Toxorhynchites inornatus* Walker , *T. brevipalpis* Theobald and *T. splendens* Wiedemann were introduced in the state of Hawai‘i. However due to intrinsic factors (i.e., low survival rates, and few offspring), their effectiveness as biological control has been limited.

Table 1.1: The current understanding of the current established distribution and records of detection of Mosquitoes species in Hawai‘i and the associated pathogens they are known to vector.

Mosquito Species	Pathogens Vectored	Established/Detected	Previously Established/ Detected	References
Aedes albopictus	DENV, CHIKV, ZIKAV, Dirofilaria	O‘ahu, Maui, Island of Hawai‘i, Kaua‘i, Lāna‘i, Moloka‘i	O‘ahu, Maui, Island of Hawai‘i, Kaua‘i, Lāna‘i, Moloka‘i	(11, 34, 35)
Aedes aegypti	DENV, CHIKV, ZIKAV, YFV	Island of Hawai‘i, Maui	Maui, O‘ahu	(11, 34, 35)
Aedes vexans	WNV, RVFV, Dirofilaria	Maui, O‘ahu	O‘ahu, Kauai	(11, 34, 35)
Aedes japonicus	WNV, JEV, LACV, EEEV, CHIKV, DENV, RVFV	Maui, O‘ahu	O‘ahu, Island of Hawai‘i	(11, 34, 35)
Culex quinquefasciatus	WNV, AM	Maui, O‘ahu, Island of Hawai‘i	O‘ahu, Maui, Island of Hawai‘i	(11, 34, 35)
*Wyeomyia mitchellii***	Unknown	O‘ahu, Maui	O‘ahu	(11, 34, 35)
*Anopheles punctipennis***	WNV	None	O‘ahu	(11, 34, 35)
Toxorhynchites brevipalpus	Unknown	O‘ahu	O‘ahu	(11, 34, 35)

* Abbreviations: West Nile virus (WNV), Japanese encephalitis virus (JEV), La crosse virus (LACV), Dengue virus (DENV), Chikunguya (CHIKV), Zika viru (ZIKAV), Eastern equine encephalitis virus (EEEV), Rift Valley fever virus (RVFV), Yellow fever virus (YFV), Dirofilaria, Avian malaria (AM)

***Anopheles punctipennis* Say; *Wyeomyia mitchellii* Theobold

Mosquitoes have played a significant role in the decline of native Hawaiian bird populations via the transmission of *Plasmodium reticulum* Gilruth, Sweet and Dodd (Haemospororida:Plasmodiidae) by *Culex quinquefasciatus* (36). This species is the primary vector for avian malaria (AM) (i.e., *Plasmodium reticulum*), is also a known vector for canine filariasis (*Dirofilaria* (Rhabditida:Onchocercidae)), and West Nile virus (WNV) (11) (Table 1.1). Avian malaria, and other infectious agents (i.e., bird pox) has caused significant impact to native Hawaiian honeycreepers, and has contributed to the declining populations (37).

In addition to their impact on wildlife, mosquitoes in Hawaiʻi have vectored disease in humans and remain a nuisance pest. The most significant vector-borne disease of humans in Hawaiʻi has been dengue fever (38). Dengue fever is caused by an arbovirus, DENV, transmitted by mosquitoes, and can cause symptoms that range from flu-like illness to hemorrhagic fever (39). The first dengue epidemic caused by mosquitoes was reported in late 1840s, and the second at the turn of the century, where an estimated 30,000 people were affected (14). Since then, there have been sporadic outbreaks of dengue fever; 2001-2002, and more recently 2015-2016 that impacted 238 people (14, 40). Other vector-borne diseases that have been detected in Hawaiʻi are chikungunya, and zika, however to my knowledge these cases have not been locally transmitted.

The primary mosquito vectors of human disease in Hawaiʻi are *Ae. Aegypti* Linnaeus*, Ae. albopictus*, *C. quinquefasciatus*, *Ae. japonicus* Theobald, and *Ae. vexans* Meigen (Table 1.1). In the outbreak of dengue fever in the 1800s, the Asian tiger mosquito, *Ae. aegypti* was widely distributed throughout the islands (14) but after significant management of mosquitoes, the population was reduced and even eradicated from some of the islands like Oʻahu. The range has contracted to only the Island of Hawaiʻi. A*edes japonicus*, is also a known vector for several arboviruses including Japanese encephalitis virus (JEV), however its distribution remains poorly understood (34). As mentioned above, *Ae. albopictus* has the potential to vector several arboviruses. The ability of this mosquito to establish in a wide variety of environments is an important element that favors its vector potential. The potential for new outbreaks is always present in Hawai'i, as there is a high probability of infected hosts moving into Hawai'i due to travel and migration. The year-round suitable climate for mosquitoes places the state at risk for vector-borne diseases year-round.

VECTOR CONTROL STRATEGIES

Effective vector control uses surveillance programs and multiple strategies to reduce mosquito populations such as: source reduction, control of immature stages (i.e., larvae and pupae), control of adults, insecticide resistance monitoring, and public education (41). Mosquito surveillance provides data that will inform vector control strategies (42). Surveillance strategies consist of identifying oviposition sites and potential larval habitats, tracking of adult mosquitoes to identify deleterious species, and creating current distribution maps to inform source reduction protocols. Furthermore, surveillance at ports of entry is critical to detecting mosquito species incursions (15). While surveillance remains the backbone of vector control programs, limited funding, and reduced staffing remain an ever-present challenge to management programs.

An effective surveillance program will increase the probability of detection of either established or newly invasive mosquitoes. Surveillance programs should incorporate a diverse suite of mosquito traps aimed at larval and adult life stages in order to effectively detect mosquito species. For instance, Center for Disease Control (CDC) designed light traps supplemented with CO_2 are effective at sampling a wide variety of mosquito species (43), while Biogents (BG) Sentinel traps were designed to capture mosquitoes of the *Aedes* genera and may not attract other types of species (43). Oviposition and gravid traps can assess if reproducing females are present, however this will not give estimate on mosquito abundance, and may require the use of different types of infusions to attract a broad range of mosquito species that have varying oviposition preferences. Thus, mosquitoes may evade detection if only one type of trap is used.

Due to budgetary restraints, Hawaiʻi vector control ceased mosquito surveillance in 2009, and resumed in 2017 after the 2015-2016 DENV outbreak on the island of Hawaiʻi. Currently, mosquito surveillance in Hawaiʻi varies by island. For instance, ports of entry and island-wide surveillance is conducted on Oʻahu and Kauaʻi (at various times of the year), while Maui monitors at ports of entry (pers. comm., Hawaiʻi Vector Control Branch). Other islands such as Lānaʻi, and Molokaʻi are understudied. The Hawaiʻi Vector Control Branch emphasizes monitoring at ports of entry and utilizes an integrated pest management approach (pers. comm., Hawaiʻi Vector Control Branch). Lack of widespread surveillance throughout outer islands such Maui, Lānaʻi, and Molokaʻi creates a vulnerability for detecting mosquito incursions, especially if mosquitoes evade detection at the ports of entry. To address this gap in knowledge, the present study seeks to determine the current distributions of mosquitoes on the island of Maui and investigate factors that influence population dynamics of the widespread mosquito *Ae. albopictus* (Chapter 2).

Mosquitoes are challenging to manage but doing so can reduce disease transmission. For example, anthropophilic mosquitoes of the genus *Aedes* are difficult to control once established, as they are able to thrive in various settings (44). Thus, using various strategies to reduce the population is essential.

Mosquitoes will oviposit and develop in standing water. One management strategy is to reduce the number of breeding sites via habitat modification. Habitat modification by establishing drainage systems is a well-known method for reducing mosquito reproduction, and thus mosquito population densities decrease as oviposition sites are reduced (10). Direct reduction of immature stages of mosquitoes can also be achieved with the use of larvicides such as *Bacillus thuringiensis* subspecies *israelensis* (Bti) Berliner (Bacillales:Bacillaceae), *Lysinibacillus spaericus* Kellen (Bacillales:Bacillaceae) and Spinosad (45), especially when oviposition sites cannot be eliminated (10).

Historically, the control of adult mosquito populations has been achieved with the use of broad-spectrum chemical insecticides such as pyrethrins/pyrethroids and organophosphastes (46) and organochlorines such as DDT. These chemicals have been used to reduce adults through insecticide treated bed nets, indoor residual spraying, direct spraying, and as topical or spatial repellants (47). These methods led to the dramatic reduction in MBDs, however overuse, and evolutionary pressure has led to a rise in insecticide resistance (48, 49), a subsequent increase in mosquito populations and an increase in MBD related deaths (48).

Newer strategies to reduce mosquito populations are being developed (50). Technologies such as CRISPR-Cas9 have shown population suppression in *Anopheles gambiae* Say mosquitoes, which are the primary vector of human malaria (*Plasmodium* spp.). Additionally, CRISPR based gene drives are being developed to target the mosquito-borne pathogens, more specifically, anti-pathogen effector genes would be spread within a mosquito vector and confer resistance toward a specific pathogen (51). While these systems offer novel approaches toward mitigating the spread of mosquito-borne diseases, there are still many barriers to implement these gene drive strategies in natural settings. A major barrier involves the mutational breakdown that occurs in gene drive strategies that results in loss of the desired gene (52, 53). Additionally, many of these gene drives rely on effector genes that are specific to a single pathogen, which greatly limits a broad scale gene drive approach to reduce mosquito-borne disease (53). There is an urgent need to develop new and innovative strategies to suppress mosquito-borne disease transmission, and recent investigations into the insect microbiome have yielded some promising tools.

MICROBIOME

The microbiome consists of diverse populations of symbiotic microorganisms that can be commensal, mutualistic, or pathogenic to the host in which they reside. The microbiome composition can influence physiological properties such as fecundity, metabolism, immunity, and disease susceptibility within a host (28). The interactions between microbiome and host, have become a new and emerging branch in entomological research. One of the pressing challenges facing microbiome studies is characterizing the variation in the microbiome over time and space in populations and among individuals. Once this variation has been described, this information can be used towards better management of insect pests.

Insects are colonized by microorganisms (bacteria, fungi, protozoans, and viruses) on a regular basis, and these symbionts account for 1-10% of the insect biomass (54). Early studies reviewing the insect microbiome paved a foundation for describing the diversity of symbionts within host species (55). Studies characterizing the microbiome of insects have increased in recent years, largely due to advances in next-generation sequencing (56). The microbiome can provide multiple benefits to the insect host including; nutritional benefits (57), tolerance to environmental fluxes (58), and even immune priming to deter predation (30). Recently, studies have shown that microbiota within insects can influence insect ecology, and reproduction (3). For example, *Wolbachia* Hertig (Rickettsiales: Ehrlichaiaceae), a genus of bacteria discovered in 1924, has been shown to kill mosquito eggs by causing cytoplasmic incompatibility (59). *Wolbachia* is now the interest of entomologists as a use for controlling insect populations and decreasing the use of pesticides. Other symbionts such as *Sodalis,* a genus of bacteria has been proposed as paratransgenic tool to block Tsetse fly (Diptera: Glossinidae) transmission of *Trypanosoma brucei* Plimmer & Bradford (Trypanosomatida:Trypanosomatidae) (African sleeping sickness) (60). *Asaia* Yamada *et al* (Rhodospirallales: Acetobacteraceae)*,* a genus of bacteria is also being investigated for use in paratrangenic platforms (61). Other symbiotic microbes are being investigated for their potential control of medically important arthropods, and these avenues of research and potential application highlight the importance of understanding arthropod microbiota.

VARIATION IN THE MOSQUITO MICROBIOME

For mosquitoes, the microbiome is essential for maintaining organismal function, more specifically immune priming, nutrition, metabolism, reproduction, development and pathogen defense (16, 21, 62). The mosquito microbiome alters the physiological traits of medically important mosquitoes, which may impact how mosquito populations sustain disease transmission (19, 26, 63). The microbiome of an individual mosquito is likely to be highly variable in time, within populations and across landscapes as they are exposed to different abiotic and biotic factors (29, 64, 65), at present ecological and environmental factors that contribute to the diversity and variation are poorly understood. Mosquitoes likely acquire the majority of this diversity from their environment (16, 64), and to some extent transstadially during metamorphosis (21, 27). Indeed, studies have shown that the immature stages of mosquitoes (i.e., larvae) have similar microbial compositions to their aquatic habitats (21) . The adult phase of mosquitoes also acquires a portion of their microbiome through environmental sources, although the full extent of this remains poorly resolved (61, 66). For instance, mosquitoes acquire the bacterial genera *Asaia* sp., through nectivorous feeding behaviors (61). Additionally, a study assessed the microbiome in pooled whole adult *Aedes aegypti*, *Culex quinquefasciatus*, and *Aedes albopictus* across different habitats in Thailand and demonstrated that while overall diversity of microbiota was low, with majority of the microbes assigned to one or a few taxa, but the proportions of microbes within the different mosquito species was highly variable (67). Overall, these studies suggest that the environment plays a role in structuring mosquito microbiotas and may be the ultimate source of its diversity.

The variability of the microbiota within mosquitoes has been highlighted in other studies (64, 68, 69). For example, Duguma *et al*., noted that there were significant differences in bacterial composition between different life-stages (larva, pupa and adult) of *Culex spp* [69.] Wang *et al*. had similar findings when looking at *Anopheles gambiae* and noted that there was a higher diversity of microbiota among larvae as compared to pupa, and adults (70, 71). The microbiome community can also vary in the tissues within a single mosquito. Characterization of mosquito microbiome thus far has focused on whole body mosquito, midgut, and to some extent salivary glands (72). The midgut, which is a site for transmittable pathogens like malaria, DENV and ZIKAV, is resident to a subset of microorganisms that are beneficial to the mosquito (54, 73). However, microorganisms that may have roles in pathogen transmission, likely reside in other tissues like the, crop, Malpighian tubules, and ovaries (74), have not been extensively explored. Additionally, microorganisms that colonize mosquitoes from environmental sources may disseminate differentially across organs and tissue types in the mosquito host. However, the mechanisms of this are unknown.

THE MICROBIOME OF <u>AEDES ALBOPICTUS</u>

In the last 5 years, major advancements have occurred in *Aedes albopictus* microbiome research (75). Culture dependent methods were initially used to isolate bacterial symbionts from

Ae. albopictus midguts (76). However, mimicking the exact physiological environments in-vitro is an ever-present challenge when cultivating mosquito symbionts. Culture-independent methods such as Denaturating Gradient Gel Electrophoresis (77, 78), taxonomic microarray hybridizations (78), High Throughput DNA Sequencing technologies (HTS), such as DNA metabarcoding (or 16S rDNA amplicon sequencing) and metagenomics (79), have allowed researchers to overcome difficulties in culture-dependent methods and assess overall bacterial diversity. Culture-independent methods have shown that that the immature stages of *Ae. albopictu*s are a subset of the aquatic breeding habitat, and that the larvae rely on microorganisms for development (21). Some studies have focused on mosquito tissues, the central focus being the midgut, salivary glands, ovaries, and testes (75). Three of the microbiome studies on *Ae. albopictus* studies have primarily interrogated the midgut of laboratory and wild-caught mosquitoes with 16srRNA sequencing technologies (67, 80, 81). Their findings have shown that the midgut of laboratory and wild caught mosquitoes differ in diversity and abundance of microbiota. Yadav *et al*., also used the first survey findings to characterize and isolate the bacterium in the genus *Dietzia* (Mycobacteriales:Dietziaceae) which is a similar to the bacterial genus *Rhodococcus* (Mycobacteriales:Nocardiaceae) (82). The fourth, a paper by Zouache *et al.*, was the first to look at the whole mosquito microbiome of wild caught *Ae. albopictus* in Madagascar (82). This study found that the bacterial composition was influenced by the environment, sex of mosquito and highlighted the importance of surveying the microbiome of wild-caught mosquitoes (81). While these studies have led to great advancements in the field of mosquito microbiome research, there are still many aspects that remain poorly resolved. Clarifying which precise environmental and ecological characteristics of geographic location that impact the structure and function of the *Aedes albopictus* microbiome is an essential area for future research and will assist in the understanding of how vectorial capacity changes across landscapes.

In the present study, these relationships are addressed through a comprehensive analysis of the microbiome of *Ae. albopictus* collected from the island of Maui over the course of a year (Chapter 3). Investigating these factors that drive variation and diversity and Hawaiʻi has many advantages. For instance, the island of Maui is a high Pacific Island (3,055 meters) that has drastic environmental gradients in monthly average temperature (~8ºC to 32ºC) and rainfall (~0.25mm to 710mm) across relatively short geographical distances (5-60 km). In addition, Maui has a mixture of land uses, from highly populated urban areas, intensive agriculture, and conserved areas such as National Parks. Thus, with these extremes in elevation, temperature and rainfall, and habitat complexity, the island of Maui can serve as a model to understand how ecological and environmental factors influence the assembly and composition of *Ae albopictus* microbiome. Furthermore, implementing novel disease reduction strategies in the field is limited by a gap in knowledge in how microbes are shared between mosquitoes in different environments and exploring this in an island population provides an ideal opportunity to do so. In this study, these relationships are addressed in *Ae. albopictus* through a comprehensive assessment of the microbiome in adult mosquitoes and environmental samples that they interact with via foraging, resting or reproductive behavior (Chapter 4).

RESEARCH OBJECTIVES

Aedes albopictus is a significant disease vector that is increasing its distribution around the world. Although it has been present in Hawaiʻi since the late 1800s (11), its distribution is not well-documented even on some of the most populous islands, such as Maui. Furthermore, the specific drivers of variation that influence mosquito population dynamics and its host-associated microbiomes are generally not well-understood for any mosquitoes including *Ae. albopictus* (25). Given the influence the microbiome has on mosquito vectorial capacity, further understanding factors that contribute to microbiome variation and diversity are extremely important (19). The primary objective of this study is to further understand drivers of variation in *Aedes albopictus* and its microbiome at various scales. This study sought to resolve the knowledge gaps by *i*) identifying the distribution and abundance of mosquitoes (and the factors influencing variation) on the island of Maui, which was previously poorly resolved (Chapter 2), and *ii*) further investigate factors which contribute to the diversity and variation in mosquito microbiotas, using *Ae. albopictus* as a model (Chapter 3 and 4). To my knowledge, this is the first study that has assessed the diversity and variation of the microbiome of a medically important mosquito in Hawai‘i. The suitable climate and island geography in Hawai‘i provides an excellent opportunity to utilize the state as a model to study drivers of variation in mosquito populations and their microbiome. Furthermore, the stark environmental gradients that occur in short geographical distance provides the opportunity to investigate abiotic and biotic factors that influence mosquito microbiomes. The unique landscape, high biodiversity, and year-round suitable climate for mosquitoes in Hawai‘i make it an excellent model system in which to study microbiomes.

Objective 1: Describe the distribution of established mosquitoes on the island of Maui in Hawai'i over the course of a year (Chapter 2).

<u>Hypothesis 1:</u> Habitats on the island of Maui range from deserts to dense rainforest and provide a diverse environment for different species of mosquitoes to establish. It is expectedthe species diversity and distribution will vary from site to site.

Hypothesis 2: Based on the ability of *Ae albopictus* to oviposit environments with minimal water sources, it is expected the species to be widely prevalent throughout sites in Maui throughout the year.

Objective 2: Evaluate the variation in the microbiome of adult *Aedes albopictus* (a) across the island of Maui and (b) between mosquitoes at each sampling location (population) over time (Chapter 3)

Hypothesis 2: The microbiota in adult mosquitoes will vary on the island of Maui over time

Hypothesis 1: The microbiota in adult mosquitoes will vary geographically across the island of Maui.

Objective 3: Clarify the environmental sources that serve as sources of mosquito symbionts in adult *Aedes albopictus*, and characterize how environmentally acquired symbionts partition among distinct tissues (Chapter 4)

Hypothesis 1: The microbiota in the gut, crop, anterior and posterior midgut, Malpighian tubules, hindgut will be different from each other.

Hypothesis 2: There will be a higher diversity of microbiota within the midgut as it is the primary site for pathogens and microorganisms.

CHAPTER 2

CURRENT DISTRIBUTION OF MOSQUITOES (DIPTERA: CULICIDAE) ON THE ISLAND OF MAUI

ABSTRACT

Mosquito-borne diseases present a global threat to public health and wildlife conservation. The introduction of mosquitoes to the Hawaiian Islands has led to arbovirus transmission in humans and wildlife. Currently, there are six blood-feeding species of mosquitoes that have been reported in Hawaiʻi , including *Aedes japonicus*, *Aedes albopictus*, *Aedes aegypti*, *Aedes vexans*, *Culex quinquefasciatus,* and *Wyeomyia mitchellii* . There is a need to survey mosquito populations and create comprehensive distribution maps that will inform vector control agencies, especially in neglected regions of the state of Hawaiʻi, such as the island of Maui. This study describes the current distribution of mosquitoes on the island of Maui in Hawaiʻi through samples collected from ovipositional and BG-Sentinel traps around the island for one year. Patterns of mosquito distribution were evaluated in relation to climate variables (rainfall and temperature), elevation, space and time. As expected, *Ae. albopictus* is abundant and widely distributed around Maui, and adult abundance and oviposition varied with site and month of collection. The data indicate that *Ae. albopictus* oviposition was positively correlated with temperature and negatively correlated with elevation and rainfall, but was associated with site. However, the abundance of adults was not similarly influenced by elevation, temperature or rainfall. *Culex quinquefasciatus* was also found to be abundant but had limited distribution as compared to *Ae. albopictus*. *Aedes vexans* and *W. mitchellii* were also collected but these species were not widely distributed or abundant. These findings provide an assessment of the island wide distribution of mosquitoes on Maui, which to my knowledge, was not previously known. Data used from this study can be used provide a baseline for future mosquito surveillance studies to

assess new mosquito species incursions, and distribution expansion of mosquitoes currently established.

INTRODUCTION

Mosquito-borne disease continues to be a threat to public health and wildlife conservation efforts (81, 83) . The threat caused by mosquitoes has increased due to expanding distribution of mosquito species, attributed to increased globalization and climate change (1, 8, 9, 84). For example, increased international travel has created a mechanism for transportation of mosquito eggs, which contributes to the spread of medically important mosquitoes to vulnerable regions of the world (85) . The arrival of West Nile Virus to the Americas in 1999 was attributed to the arrival of an infected bird or mosquito, and quickly became widely established in the United States (8). Globalization has also been attributed to the establishment of medically important mosquitoes world-wide including Europe, Brazil, and tropical islands like Hawaii (9, 11, 12, 14). While distribution of mosquitoes and MBDs remains a global problem, tropical and subtropical islands are particularly vulnerable to the mosquito incursions and MBD outbreaks due to the year-round suitable climate, frequent cargo shipments, and tourism (85-87). Continual surveillance to understand the changing distribution and detect new incursions of mosquitoes on islands is increasingly important to respond to outbreaks of human and wildlife disease. Nevertheless, even in Hawa‘i, there is little known about the distribution of mosquitoes especially on some of the less populous islands such as Maui.

The first introduction of mosquitoes to Hawai‘i occurred in the end of the 17th century via cargo shipment and there have been subsequent incursions resulting in currently eight introduced mosquito species reported in the state of Hawai‘i : *Aedes albopictus*, *Aedes aegypti* *Aedes vexans, Aedes japonicus*, *Culex quinquefasciatus*, *Wyeomyia mitchellii* , and

Toxorhynchites brevipalpis and *Toxorhynchites splendens* with 5/8 of these being competent vectors of human and animal pathogens (11). All of these mosquitoes with the exception of *Toxorhynchites* sp. have been accidently introduced to Hawaiʻi and in association with human activities. The likelihood that more species incursions will occur is high. For instance, *Anopheles punctipennis* Say almost established in 2003 (88).

As global mean air temperatures have risen and are expected to increase by 1.8 to 5.8 ºC by the end of the century, mosquito distribution and transmission of MBDs is likely to increase in these new and vulnerable regions because mosquitoes generally favor warmer temperature and many species are limited in distribution and population growth by cooler temperatures. Thus, climate change and globalization may drive MBD emergence by augmenting the distribution of medically important mosquitoes.

Surveillance programs are conducted on islands in the State of Hawai'i by Federal and State agencies to monitor incoming mosquito species at points of entry such as seaports and airports and at individual government installations. As new species are detected, action is taken to prevent establishment. While this work is critical to preventing new species establishment, ongoing monitoring in other areas at different elevations and habitats provides important information regarding the current distribution and abundance of mosquitoes throughout the state of Hawaiʻi. Comprehensive distribution and abundance maps would provide further information for state agencies and allow them to act quickly in the event of a MBD outbreak. To my knowledge island-wide distribution maps have not been created for Maui, Molokai or Lānaʻi. In this study, I document the current distribution and abundance of mosquitoes on the island of Maui, Hawaiʻi over the course of a single year through the deployment of ovipositional and adult traps. The findings are analyzed with respect to elevation, temperature and rainfall as it changes across the sampled sites and over time. The findings provide a baseline for future surveys and to evaluate risks in relation to MBD and climate change.

METHODS

Mosquito eggs and adults were collected at various sites around the island of Maui at two-month intervals across the island of Maui from April 2017 to February 2018 (Table 2.1). Sites were selected to attempt to capture mosquito distribution across the entire island of Maui, and distinct habitats that ranged in microclimates and elevations (Table 2.1). The sites selected were unknown regarding the capacity to support mosquitoes. Oviposition traps at were placed opportunistically at Kahului Airport (KAH-AIRPT) following a detection of *Ae. aegypti* for two sampling periods only (December 2017 and February 2018).

Average temperature and rainfall measurements were acquired from each site and collection period from the Climate Atlas of Hawaii (Appendix A). These sites ranged in altitude from 18 to 975 m and spanned monthly rainfall and temperature gradients of 2.29 to 271.4 mm and 15 to 26°C respectively (Appendix A).

Mosquito collection

Mosquito eggs were collected at 19 sites (Table 2.1) from standard oviposition traps. To construct the oviposition trap, a 1 L black plastic container (Home Depot, Atlanta, Georgia) was filled with one of two types of water either 1-liter (L) of fresh water or grass infused water. The water used for fresh, and grass infused traps was aged by placing filtered fresh water (Costco, city, Washington) in a standard plastic bucket made with High Density Polyethylene for 14 days. Half of the aged water was used for the freshwater traps and the other half was used for the grass infused traps. Grass infused water was prepared by placing 50 grams of St. Augustine grass in 18

L of fresh filtered water in the same style standard plastic bucket used above (adapted from Tarrant County Public Health Department, http://health.tarrantcounty.com)

The grass was obtained from fresh lawn clippings at the Kula Research Station on Maui. The grass infusion was allowed to ferment with covered with cheese cloth (Sceng, California) at ambient temperatures for 10 days. Fresh-water traps were also allowed to "ferment" covered at ambient temperatures for 10 days. The fresh and grass-infused water were transported and then decanted in the trap containers at each site. Each oviposition trap (fresh and grass-infused) had a textured wooden paddle (Nelson Wood Shims, Cohasset, Minnesota) secured vertically on the wall of the container with a 1 ¼ binder clips (ACCO, China) to serve as a roost and site for oviposition.

An oviposition trap of each type (fresh and grass-infused) was placed at each of the 19 sites and left at each site for 3 days. The traps were removed, the water was filtered, and any mosquito eggs were counted and placed into mosquito rearing chambers (BioQuip Products, Rancho Dominguez, CA, USA) with fresh water and fed every two-days with Gold-Fish Flakes (TetraFin, Germany). Once larvae reached the 4th instar they were identified to species using the Darsie and Ward taxonomic key (89). Eggs that did not hatch were discarded after two-weeks.

Adult mosquitoes were collected at nine sites (Table 2.1) across the island of Maui, Hawaiʻi at two-month intervals over the course of a single year (April 2017 to February 2018). Adult mosquitoes were collected with BG Sentinel Traps (Bioagent's, Rogensburg, Germany), powered with 12 Volt, 21 Amp Hour batteries (Powersonic, San Diego, CA) and baited with approximately 1 kilogram of dry ice as a source of CO_2 (Airgas, Hawaii) and BG lure (BioQuip, Rancho Dominguez, California). A single adult trap was placed once at each site for 24 hours. The traps were placed on site on one day, then serviced at the same time the following day and moved to another site for the next sampling period. The sampling of the 9 sites occurred over three days. After the 24-hour sampling at each site, all mosquitoes were removed from the trap and identified to species and sex using a standard 10-40X dissecting scope and the Darsie and Ward taxonomic key (89).

STATISTICAL ANALYSIS

Of the mosquitoes collected only Ae. albopictus was recovered from either oviposition or adult traps in sufficient numbers to enable statistical analysis. The oviposition traps placed at Kahului airport on only two occasions were excluded from statistical analysis. On three occasions (June: Fresh water trap at Haleakala Road 2 and grass-infused trap at Kaumahina State Park; December: fresh water trap at Olowalu) one of the traps at one of the sites was tipped over and no samples were obtained.

Eggs/larvae

The presence/absence rather than the abundance of eggs of a particular species of mosquito was analyzed from the oviposition traps, for the following reasons. First, a single female mosquito will lay multiple eggs, between 1-300 depending on species and she may lay these in batches of varying size and in different locations (90). For example, some gravid females such as *Ae. aegypti* and *Ae. albopictus* will seek to increase reproductive potential by exploiting habitats with diverse nutrient richness and will deposit their eggs in multiple locations with a behavior known as “skip oviposition” (90-92). Multiple females may also lay eggs in a single container. Additionally, gravid females at times will use the presence or absence of mosquito eggs to assess a potential oviposition site (93). Thus, when evaluating the number of eggs in a container, I cannot rule out one or multiple oviposition events from one or multiple females. As such, counts are not reliable indicators of abundance. Subsequently, presence-absence data rather than counts of egg or larvae was utilized in statistical analysis.

The variation in presence or absence of *Ae. albopictus* eggs across site and month of collection was analyzed using a generalized linear model with a binomial error distribution (package “MASS” in program R). Trap type was used as a covariate to account for variation between fresh and grass-infused trap types. The effect of site and month on the presence or absence of *Ae. albopictus* eggs was assessed through a log-likelihood ratio test that compared a full model and a nested model that lacked an independent variable of interest. I assumed the log-likelihood approximates a Chi-square distribution.

Climate variables (rainfall, temperature) and elevation from 18 sites were related to the presence or absence of *Ae. albopictus* eggs through a two-step process (98). First, a Principal Component Analysis (PCA) was used to resolve autocorrelation among the climate variables and elevation. Then the variation in presence or absence of *Ae. albopictus* oviposition in response to the variables PCA scores was analyzed using generalized linear mixed models assuming a binomial error distribution (package *glmmTMB* in program R). All models incorporated a site and month random effect to control for spatiotemporal variation. The significance of individual independent fixed effects on the presence or absence of *Ae. albopictus* eggs was evaluated through a log-likelihood ratio test that compared a full model and a nested model that lacked the independent variable of interest. I assumed the log-likelihood approximates a Chi-square distribution.

<u>*Adult abundance*</u>

The variation in abundance of *Ae. albopictus* across site and month was analyzed using a generalized linear model assuming a negative binomial (“nbinom1”) error distribution (package

“MASS” in program R). The influence of site and month on *Ae. albopictus* abundance was assessed through a log-likelihood ratio test as described above. Pairwise comparisons of *Ae. albopictus* between sites and months of collections were assessed with “pairwise.t.test” with a Bonferroni correction implemented in package “stats” in R.

Climate variables and elevation from the 9 sites from which adults were collected were related to *Ae. albopictus* abundance using generalized linear mixed models assuming a negative binomial (“nbinom1”) error distribution (package *glmmTMB* in program R). A similar process was used to test for relationships between in *Ae. albopictus* abundance and climate variables and elevation as described above.

Table 2. 1: Sites across the Island of Maui where traps to collect either mosquito eggs after oviposition (O) or adults (A) were placed at two-month intervals for one year from April 2017-February 2018. One site (Kahului airport) had traps placed only two occasions, once in December and February.

Site Name	Abbreviation	Latitude	Longitude	Elevation (m)	O	A	Proximity to Human Habitation	Life Zone	Vegetation noted at Sites
Haleakala National Park	HAL	20.767487	-156.2413	2051	Y	N	Limited	Inland, subalpine dry scrub	Scrub bushes
Haleakala Road 1	HAL 1	20.76264	-156.28824	1443	Y	N	Limited	Inland, subtropical, lower montane, moist forest	Grassland with scrub bushes
Haleakala Road 2	HAL 2	20.76689	-156.302	1131	Y	N	High	Inland, subtropical, lower montane, moist forest	Castor oil plants and Oak tree
Hana Health	HAN	20.763534	-155.99442	63	Y	Y	High	Coastal, subtropical wet forest	Banyan trees, and date palm
Hana Hwy 1	HAN 1	20.881008	-156.2042	234	Y	N	Limited	Coastal, subtropical wet forest	Various shrubs, strawberry guava,

Kahului (KAH)	KAH	20.889865	-156.47573	20	Y	Y	High	Coastal, subtropical thorn woodland	Maintained nursery
Kahului Airport	KAH-AIRPT	20.8924	-156.424	19	Y	N	High	Coastal, subtropical thorn woodland	Agriculture sugarcane, various shrubs
Kaumahina State Park	KAUM	20.871663	-156.16936	136	Y	N	Low	Coastal, subtropical wet forest	Banyan, Castor oil plants
Kaupo	KAUP	20.63463	-156.13658	59	Y	Y	Limited	Subtropical moist forest surrounded by perennial grassland	Persian silk trees, grassland
Kealia Pond	KEA	20.795928	-156.48574	20	Y	Y	Low	Coastal, subtropical desert scrub	Kipukai (shrub), \`uhaloa (shrub)
Keokea	KEO	20.70573	-156.35677	844	Y	Y	High	Inland, subtropical lower montane moist forest	Cape ivy, Persian silk trees
Kipahulu	KIP	20.6529	-156.0572	92	Y	N	High	Coastal, subtropical wet mesic forest	Date palms, banyan trees
Kula	KU	20.757617	-156.32103	976	Y	Y	High	Inland, subtropical, lower montane dry forest	Avocado trees, bromeliads
Lahaina	LAH	20.890853	-156.68395	19	Y	N	High	Coastal, subtropical thorn woodland	Palms, scrub bushes

Maui Forest Bird Recovery	MFBR	20.806919	-156.2804	1153	Y	Y	Low	Inland, subtropical, lower montane, wet forest	Dense vegetation with various types of trees
Maui Invasive Species Council	MISC	20.840894	-156.29374	660	Y	Y	High	Inland, subtropical, lower montane, wet forest	Palms, Oak trees
Olowalu	OLO	20.811112	-156.62107	20	Y	Y	Low	Coastal, subtropical, desert scrub	Scrub bushes
Waihee	WAI	20.958035	-156.52969	262	Y	N	Low	Coastal, subtropical, moist forest	Grassland, Norfolk pine trees
Wakina	WAK	20.928694	-156.31458	89	Y	N	High	Coastal, subtropical moist forest	Banana Tree, Castor oil plants

* Sites HAL, HAL1, HAL2, KEO, KUL, MFBR, MISC, AND WAI were in mountainous areas

RESULTS

<u>Four mosquito species detected on the island of Maui, Hawai'i</u>

Over the course of this study, a total of 1016 mosquitoes were collected across the Island of Maui, Hawaiʻi . Of the 736 eggs that were collected, 686 larvae hatched (93% hatch rate) yielding two mosquito species: *Ae. albopictus* (14%) and *Cx. quinquefasciatus* (86%). While a greater number *Cx. quinquefasciatus* larvae hatched, this mosquito species was captured in only 3/6 collection periods, and at 5/19 sites. Most mosquito eggs were captured at Lahaina (LAH, 65%) and Maui Forest Bird Recovery (MFBR, 17%) and the resultant larvae were predominately *Cx. quinquefasciatus* (LAH: 100%, MFBR: 100%; Table 2.3). In contrast, *Ae. albopictus* eggs were detected in lower abundance but more frequently 5/6 collection periods and 10/19 sites (Table 2.2 and 2.3).

Table 2.2: The sum of mosquito larvae and total number of *Aedes albopictus* (AA) and *Culex quinquefasciatus* (CQ) that hatched from eggs collected at all sites with each sampling period from April 2017 to February 2018.

Month	AA	CQ	Sum
April	8	0	8
June	17	211	228
August	22	350	372
October	5	37	42
December	35	9	36
February	0	0	0
Sum	87	607	694

*AA=*Aedes albopictus*, CQ=*Culex quinquefasciatus*

Table 2.3: The total number of *Aedes albopictus* (AA) and *Culex quinquefasciatus* (CQ) and total number of all mosquito larvae that hatched from eggs collected at each site where oviposition traps were placed across all sampling periods.

	Sites																		
	HAK	HAK1	HAK2	HAN1	HANH	KAH	KAH-AIRPT	KAUM	KAUP	KEA	KEO	KIP	KUL	LAH	MFBR	MISC	OLO	WAI	WAK	Sum
AA	0	0	0	9	22	0	0	10	2	0	1	7	0	0	0	2	2	3	29	87
CQ	0	0	0	0	30	0	8	1	0	1	0	0	0	451	116	0	0	0	0	607
Sum	0	0	0	9	52	0	8	11	2	1	1	7	0	451	116	2	2	3	29	694

* Site Abbreviations: Haleakala National Park (HAK), Haleakala Road 1 (HAK1), Haleakala Road 2 (HAK2), Hana Hwy 1 (HAN1), Hana Health (HANH), Kahului (KAH), Kahului Airport (KAH-AIRPT), Kaumanhina State Park (KAUM), Kaupo (KAUP), Kealia Pond (KEA). Keokea (KEO), Kipahulu (KIP). Kula (KUL), Lahaina (LAH), Maui Forest Bird Recovery (MFBR), Maui Invasive Species Council (MISC), Olowalu (OLO), Waihee (WAI), and Wakina (WA

Four species were identified from the 322 adults that were collected: *Ae. albopictus* (92%), *Cx. quinquefasciatus* (4%), *W. mitchellii* (3%), and *Ae. vexans* (1.2%), with *Ae. albopictus* (92% of adults) accounting for most of the adult mosquitoes captured (Appendix B and S3). A vast majority of adult mosquitoes were collected in the months of June (42%) and October (19%), with a high proportion identified as *Ae. albopictus* (June: 95%, October: 95%; Appendix B). Fewer mosquitoes were captured in December (5%) and February (9%). *Aedes albopictus* was always collected at all sites and across Maui, given its abundance and distribution, the factors influencing the variation in *Ae. albopictus* are presented in more detail in the following section. Summary results about the other species are presented below.

All four species were collected from only two sites on the Island, Kealia and Maui Forest Bird Recovery (Table 2.1), which differ in elevation, and habitat type (Table 2.1), Mosquito species *W. mitchellii* and *Ae. vexans* were captured in-frequently, and only with adult traps. Nine individuals of *W. mitchellii* were collected from adult traps over three sites (Kealia, Maui Forest Bird Recovery, and Maui Invasive Species Council) and on three occasions (Appendix B and C). Only four individuals of *Ae. vexans* were collected during this study: one in April at Maui Forest Bird Recovery and three at Kealia in June 2017. It is also possible that *Ae. vexans* and *W. mitchellii* eggs were among the 7% of eggs that did not hatch.

Adult *Cx. quinquefasciatus* was present throughout the year in lower abundance relative to *Ae. albopictus* and was captured at 5/9 sites (Appendix B and C). Only 14 adults were captured at four sites (Hana Health, Kahului, Maui Forest Bird Recovery, and Olowalu), and were detected in all sampling months except for August. A total of 599 *Cx. quinquefasciatus* larvae were detected in 4/6 sampling periods (Table 2.2) at 6/19 sites. The majority of *Cx.*

quinquefasciatus were detected from two sites that ranged in elevation and habitat type. For instance, 19% of *Cx. quinquefasciatus* were collected at Maui Forest Bird Recovery, while 74% were collected at Lahaina (Table 2.1). Generally, *Cx. quinquefasciatus* was detected in diverse habitats (for example at Lahaina: elevation= 18 m, mean rainfall = 29 mm, mean temperature = 29 ^{o} C; Maui Forest Bird Recovery: elevation = 1153 m, mean rainfall = 154 mm, mean temperature = 16 ^{o} C) that vary in mean rainfall and temperature, and elevation (Table 2.1).

<u>*Aedes albopictus oviposition varies with site, month of collection, and climate variables*</u>

Aedes albopictus oviposition varied with site (p-value = 0.014), and month (p-value =0.05). This effect was likely driven by changing environmental variables at the different sampling events and sites. The principal components of climate variables (rain, temperature) and elevation from 18 sampling sites in relation to the presence or absence of eggs indicated that principal component 1 (PC1), which explained 61% of variance, had positive loadings on temperature and negative loadings on elevation and rainfall. The PC1 was largely driven by temperature and elevation, while rainfall had moderate effect. Thus, a highly positive PC1 score represents a warm, low elevation site, while a negative score represents either *i*) a cool, high elevation site or *ii*) a rainy, high elevation site (Table 2.4). PC2, which explained 34% of variance, had positive loadings on elevation, and negative loadings with rainfall. Thus, a positive score represents a dry. While a negative score represents a rainy (Table 2.4, Figure 2.1). *Ae. albopictus* oviposition was positively associated with Component 1 (p value = 0.03; Table 2.5), and negatively associated with Component 2 (p value = 0.014; Table 2.5, Figure 2.1). Further corroborating this finding, *Ae. albopictus* oviposition events were not detected in elevations greater than 660 m and was consistently detected in sites with low elevations (range: 63-234 m).

Table 2.4: PCA summary and findings for climate variables relating to the presence or absence of *Aedes albopictus* eggs collected from 18 sites where oviposition traps were placed.

Climate Variable (Proportion of Variance)	PC1 (0.61)	PC2 (0.34)	PC3 (0.05)
Temperature	0.709		0.705
Rainfall	-0.130	-0.978	0.166
Elevation	-0.693	0.209	0.690

*Loadings for oviposition data

Table 2.5: The estimates and standard errors of fixed effects and the variances and standard deviation of the random effects in a mixed effect model that interrogates the probability of detecting *Ae. albopictus* oviposition events between sites and months of collection.

Full Model	Estimate	Standard error
Intercept	-2.0	0.37
Component 1	0.64	0.29
Component 2	-0.64	0.26
Trap Type	-3.1	1.1
Conditional Model		
Random Effects	Variance	Standard Deviation
Site	1.2×10^{-8}	0.00011
Month	6.77×10^{-2}	0.25

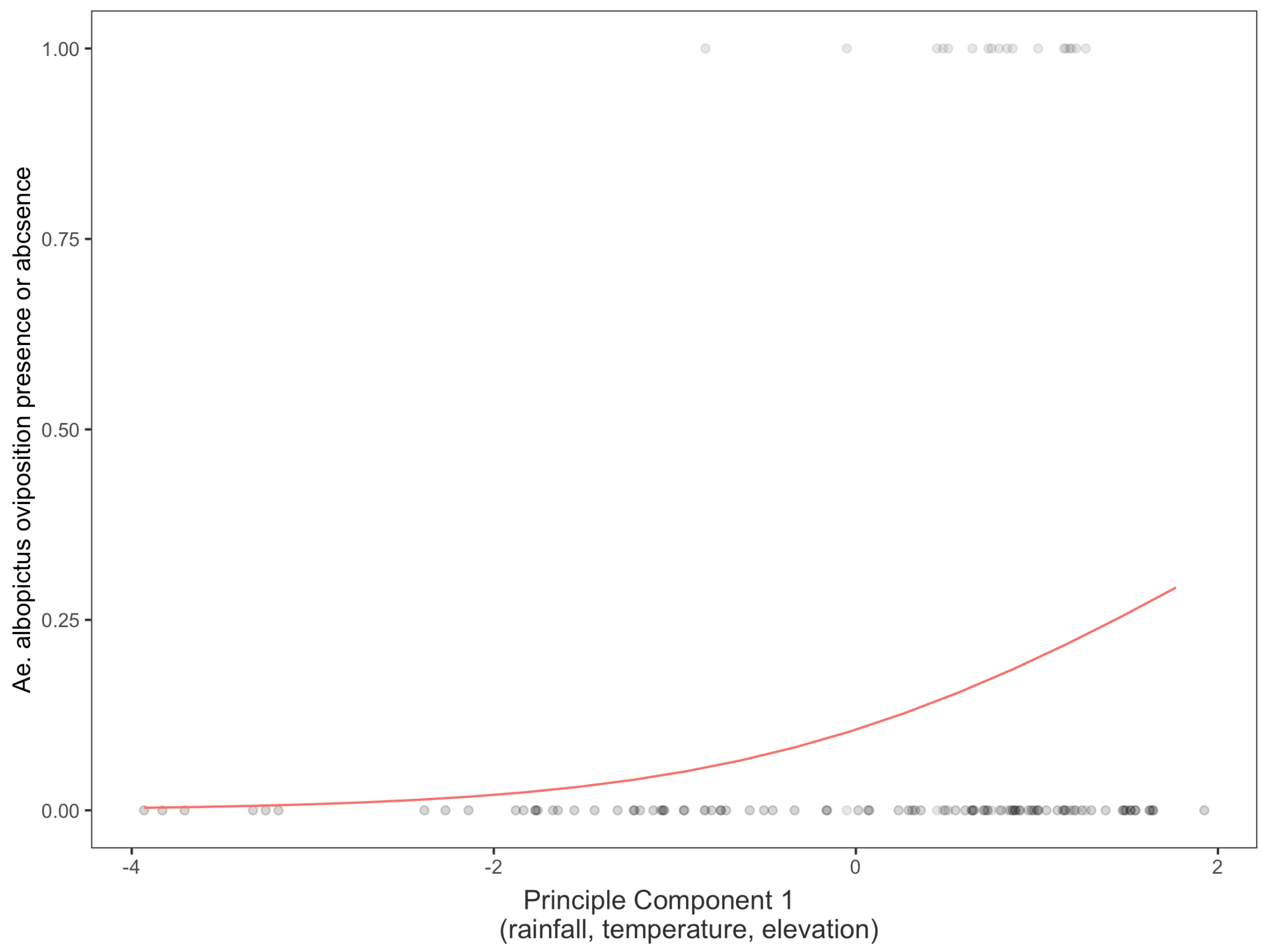

Figure 2.1: Relationship of *Ae. albopictus* oviposition to Component 1 scores for across the sampling period analyzed. The line is the predicted relationship derived from the best fit model (Table 2.5). The dots represent presence of Ae. albopictus eggs at sites. Oviposition of Ae. albopictus is positively correlated with temperature (positive Component 1 values) and negatively correlated with elevation and to a lesser extent rainfall (negative Component 1 values)

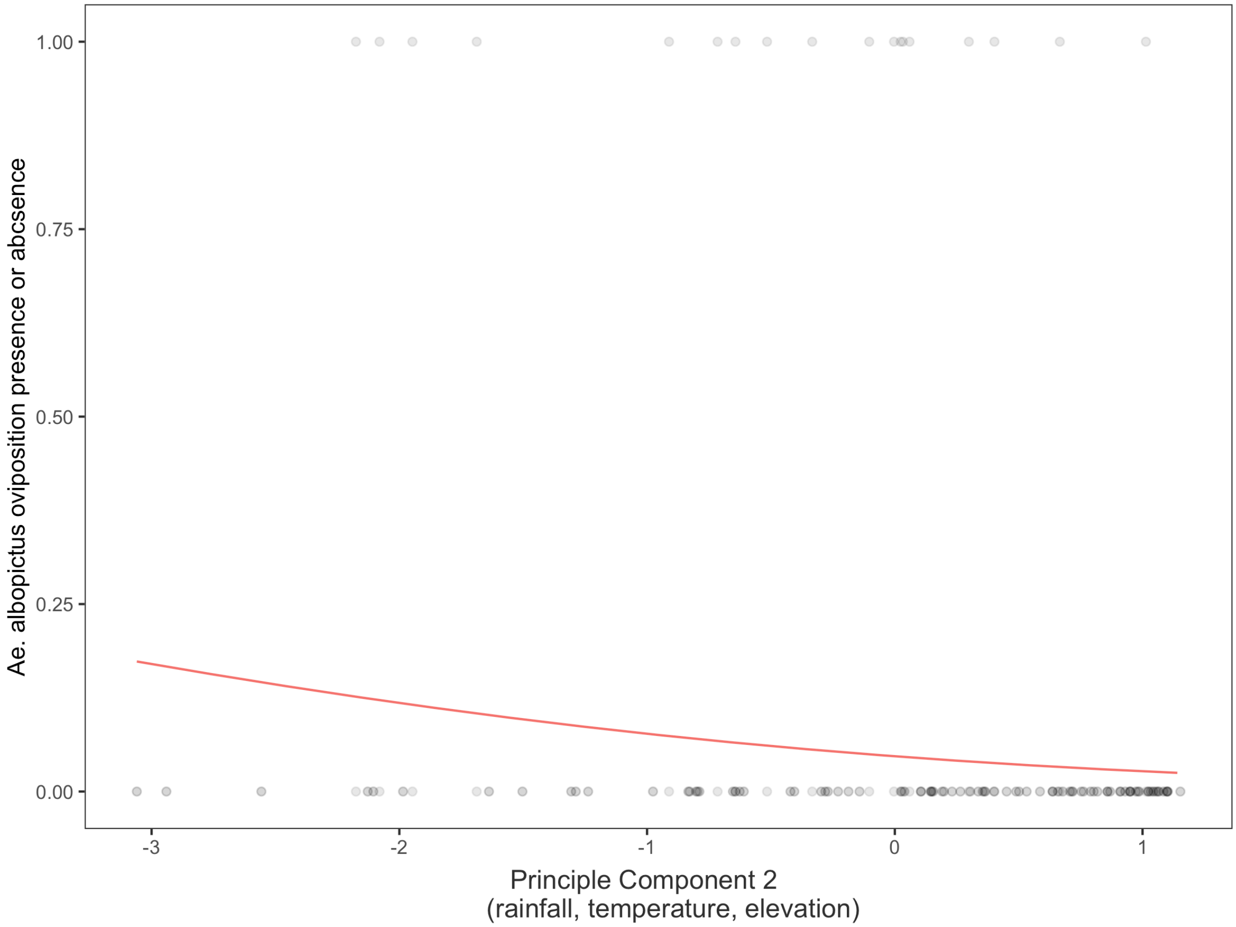

Figure 2.2: Relationship of *Ae. albopictus* oviposition to Component 2 scores for across the sampling period analyzed. The line is the predicted relationship derived from the best fit model (Table 2.5). The dots represent presence of *Ae. albopictus* eggs at sites. Oviposition of *Ae. albopictus* is negatively associated with dry sites (negative Component 2 values) and positively associate with rainy (positive Component 2 values).

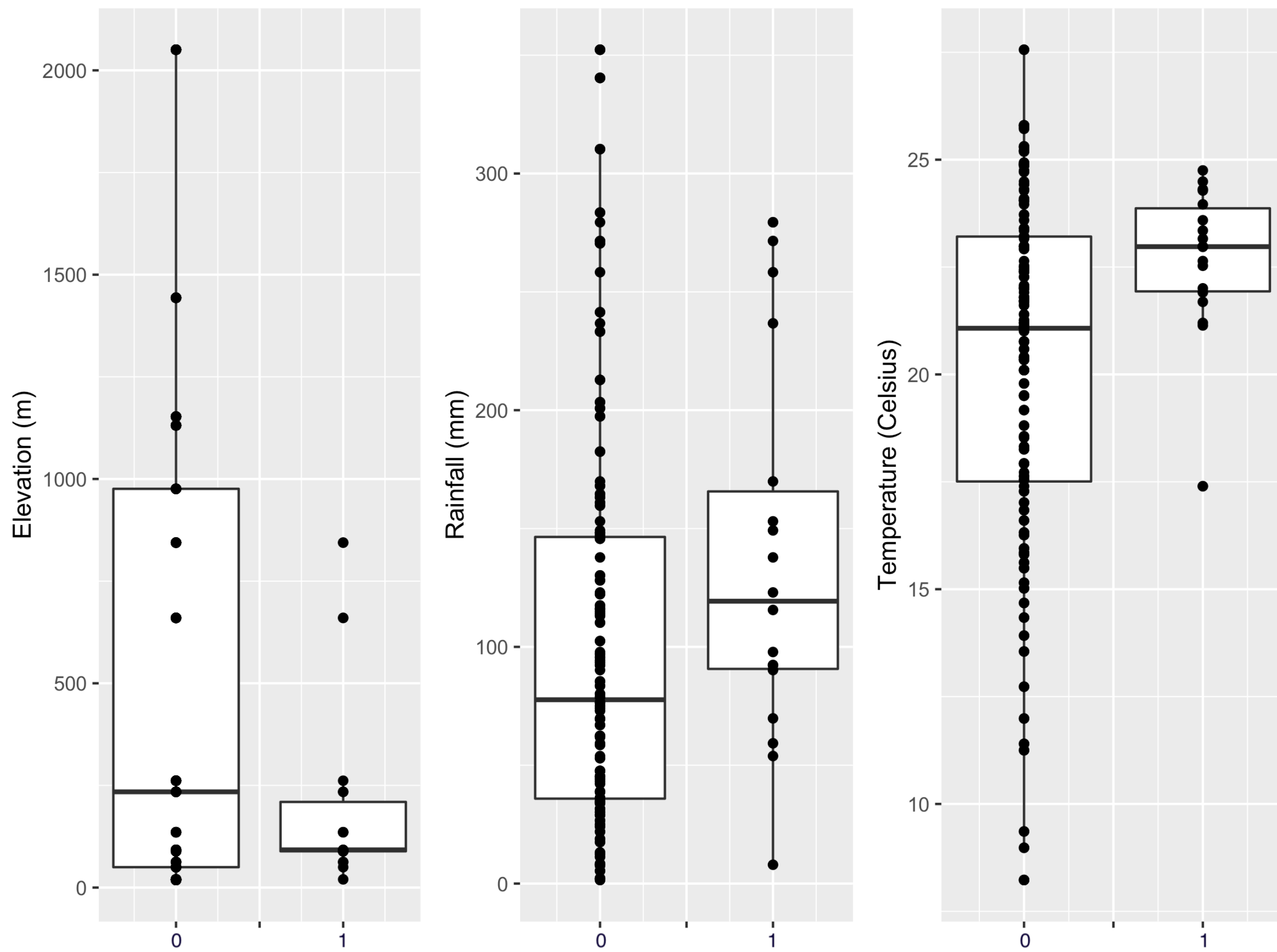

Figure 2.3: Presence or absence of *Ae. albopictus* eggs collected throughout at all sites with each sampling period from April 2017 to February 2018 and depicted in relation to temperature, rainfall, and elevation.

<u>Aedes albopictus abundance varies with month of collection and site</u>

The abundance of adult *Ae. albopictus* was found to be influenced by site (p value <0.001; Table 2.7, Figure 2.4) and month of collection (p value = 0.0005; Table 2.7, Figure 2.5). The site Hana Health (Table 2.1) had significantly different values from sites Maui Invasive Species Committee, Olowalu, Kaupo, Maui Forest Bird Recovery, and Kealia Pond. Site Kahului had the second highest mean capture rate and was significantly different from Keokea and Kealia (Figure 2.4). The higher numbers of mosquitoes in June (mean capture rate=14) are likely to be the main driver for the effect of month on adult abundance (Appendix A, Figure 2.5). Indeed, mean capture rate of *Ae. albopictus* in June was significantly different from April, August, December, and February (Figure 2.5).

The principal components of climate variables collected from 9 sites in relation to the abundance of *Ae. albopictus* adults indicated that PC1, which explained 61% of the variance, had positive loadings on temperature and negative loadings on elevation and rainfall. Thus, a highly positive PC1 score represents a warm, low elevation site, while a negative score represents either *i*) a cool, high elevation site or *ii*) a rainy, high elevation site (Table 2.6). PC2, which explained 34% of variance, had positive loadings on rainfall and temperature, and negative loadings with elevation. Thus, a positive score represents a warm, rainy site, while a negative score represents a dry high elevation site. While site and time influenced *Ae. albopictus* abundance, the climate variables associated with each site are not statistically influential when taking site and time into account (PC1 (p value = 0.23; Table 2.6 and 2.7) and PC2 (p value = 0.37; Table 2.6 and 2.7).

Table 2.6: PCA summary and findings for climate variables collected from 9 sites where adult traps were placed.

Climate Variable (Proportion of Variance)	PC1 (0.61)	PC2 (0.34)	PC3 (0.05)
Temperature	0.643	0.200	0.739
Rainfall	-0.469	0.866	0.173
Elevation	-0.606	-0.458	0.651

*Loadings for Adult *Ae. albopictus* abundance data.

Table 2.7: The estimates and standard errors of fixed effects and the variances and standard deviation of the random effects in a mixed effect model that interrogates the change in Ae. albopictus abundance between sites and month of collection.

Full Model	Estimate	Standard error
Intercept	1.26	0.42
Component 1	0.26	0.22
Component 2	-0.36	0.40
Conditional Model		
Random Effects	Variance	Standard Deviation
Site	0.93	0.96
Month	6.12×10^{-9}	7.8×10^{-5}

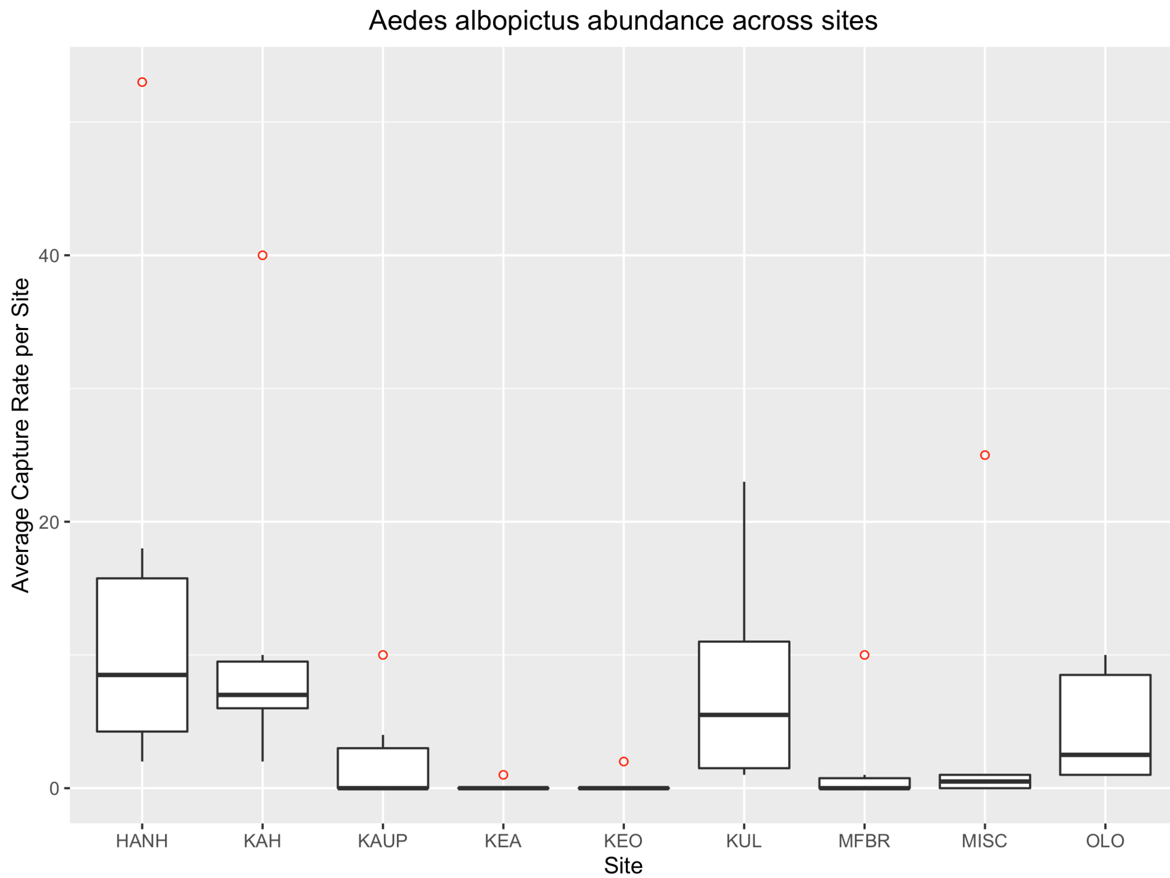

Figure 2.4: Differences in average capture rate of adult *Ae. albopictus* mosquitoes per site throughout the April 2017 to February 2018 sampling period. Pairwise comparisons of Ae. albopictus between sites collections were assessed with "pairwise.t.test" with a Bonferroni correction implemented in package "stats" in R. Only significant p-values <0.05 are shown. The following describes the differences between two sites: HANH and MISC (p value = 0.048), HANH and OLO (p value =0.048), HANH and KAUP (p value = 0.019), HANH and MFBR (p value = 0.015), HANH and KEA (p = 0.0069), KAH and KEO (p value = 0.036), KAH and KEA (p value= 0.034). The red dots are outliers.

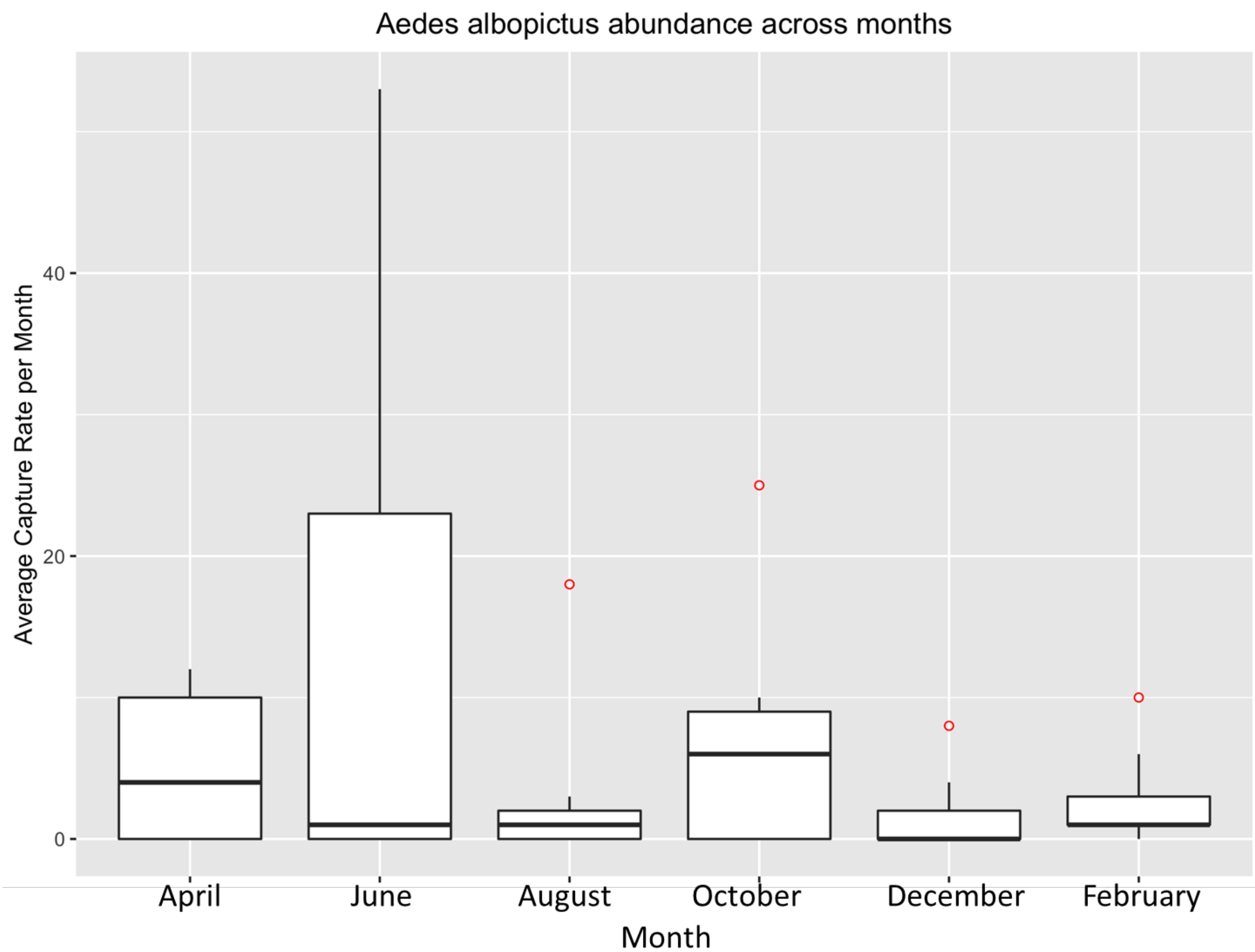

Figure 2.5. Differences in average capture rate of adult *Ae. albopictus* mosquitoes per month throughout the April 2017 to February 2018 sampling period. Pairwise comparisons of Ae. albopictus between sites collections were assessed with “pairwise.t.test” with a Bonferroni correction implemented in package “stats” in R. Only significant p-values <0.05 are shown. The following describes the differences between two months: April and June (p value = 0.046), August and June (p value = 0.016), February and June (p value = 0.014), December and June (p value = 0.008). Red dots indicate outliers.

DISCUSSION

To my knowledge, this is the only island-wide survey of mosquitoes that has been conducted systematically across the island of Maui. This study documents the occurrence of four mosquito species on the Island of Maui, *Aedes albopictus*, *Aedes vexans*, *Culex quinquefasciatus*, and *Wyeomyia mitchellii*. *Aedes albopictus* is widely distributed and abundant over much of the island and present year-round, and its abundance (i.e., adult capture) and oviposition (i.e., presence or absence of eggs) was influenced by space (site) and time (month). The results demonstrate that the spatiotemporal variation of *Ae. albopictus* oviposition is influenced by climate variables (temperature and rainfall) and elevation. These results contribute to understanding mosquito population dynamics in relation to habitat, environmental gradients and can inform vector control strategies by identifying areas that will support mosquito oviposition.

This study shows that abundance of *Ae. albopictus* adults varies with site and month of collection but unlike the presence/absence of oviposition was uncorrelated with climate variables (rainfall and temperature) and elevation. While PC1 and PC2 were not significantly associated with adult *Ae. albopictus* abundance, the differences associated with site were likely associated with drastic differences in habitats (Table 2.1). These sites represent distinct habitats, Hana Health is described as a subtropical wet forest that is extremely proximal to human habitation as it was located near a health clinic (Table 2.1). The site at Hana Health was abundant in oviposition sites (e.g., artificial containers, tree holes, areas that pool water) that support the reproduction of this mosquito species. In contrast, the site at Kealia Pond is a coastal subtropical desert scrub salt near the South-Central coast of Maui and is not proximal to human habitation (Table 2.1) (40). While Kealia Pond is not proximal to human habitation, this wildlife refuge is open to the public. However, the lack of oviposition sites due to the dry climate, little to no artificial containers, or plants that pool water, and occurrence of natural predators (e.g., dragonflies) is likely why only one individual was detected at Kealia. It is likely that natural predators (i.e., dragonflies, *Toxorhynchites sp*.) were located at other locations, however dragonflies were only noted at Kealia Pond.

Other sites also varied in mean capture rate of *Ae. albopictus*, such as Kahului and Kula. While both sites are distinct in climate types and elevation (Table 2.1), the proximity to humans and presence of oviposition sites (both artificial and natural) are similar. The site at Kahului had more artificial containers (e.g., plant pots, areas that pool water) as it was located in a commercial nursery, as compared to Kula, which is surrounded by natural oviposition sites (e.g., tree holes and bromeliads). Therefore, a lack of correlation between climate variables and *Ae. albopictus* abundance could be that the climate variables do not capture the complexity of the habitat at any one site or time. Differences in *Ae. albopictus* abundance may be attributed to the complex factors described above such as proximity to human habitation, presence of sites that support oviposition (artificial or natural), or presence of natural predators (dragonflies and/or *Toxorhynchites sp.*). For instance, a mathematical model investigating the impacts of environmental factors on mosquito dispersal found that *Ae. albopictus* was strongly influenced by available vegetation that is capable of supporting oviposition (94). Additionally, the differences in abundance may be attributed to biotic factors such as microbiome differences in distinct habitats that mosquitoes are exposed to via interactions with environmental sources through foraging, host-seeking, resting, and reproductive behavior (17, 64, 95).

In contrast to *Ae. albopictus* abundance, these results demonstrate that the occurrence of *Ae. albopictus* oviposition events shift in accordance with local climate variables (temperature and rainfall) and elevation. To my knowledge, this is the first study to assess all these factors (temperature, rainfall, and elevation) in relation to *Ae. albopictus* oviposition and abundance in one study. These data suggest that oviposition (as indicated by the presence of *Ae. albopictus* larvae) is driven primarily by warm low elevation sites and less so by rainfall (Figure 2.1). The sites in which *Ae. albopictus* oviposition were detected were all warm sites (temperature range: 19.3-23.1^{o}C) with a broad range of elevations (elevation range: 20-844 m). Although the elevation ranges were broad, *Ae. albopictus* oviposition was detected more frequently at low elevation sites (Table 2.1: Wakina 4/6 sampling periods, Kaumahina State Park 3/6 sampling periods) as compared to high elevation sites (Table 2.1: Maui Invasive Species Council 1/6 sampling periods, Keokea 1/6 sampling periods).

Previous studies have also described environmental influence with mosquito occurrence e.g., temperature, rainfall, elevation. For instance, a recent study described the influence of ecological factors on mosquito abundance and occurrence in the Galápagos islands, specifically *Ae. taeniorhynchus* Weidemann and *Cx. quinquefasciatus* decreased in abundance with elevation (100). Results from my study differ from a previous study that found no effect with factors (temperature, humidity, and rainfall) in relation to *Ae. albopictus* mean egg density, total amount of eggs, and presence of eggs in oviposition traps among sampling events (96). The pattern in the data suggest a positive association with detection of *Ae. albopictus* oviposition and rainfall and indicate that a change in the overall presence of oviposition sites at each location. Indeed, studies have shown that immediately following a rainfall event, there are marked changes in ammonium ($NH4+$) concentrations, which impact microbial communities, and nutritional resources which are important for mosquito larval development (97). Additionally, an increase in rainfall would produce a greater abundance of natural oviposition sites from which females may choose to oviposit, potentially reducing the desirability of the ovipositional traps in this study

Furthermore, oviposition sites in which *Ae. albopictus* was detected also varied in proximity to human habitation, and available vegetation, which may have influenced the spatiotemporal variation demonstrated (Table 2.1). Sites with little to no proximity to human habitation included Hana Hwy 1, Kaupo, and Kipahulu (Table 2.1). These sites also had differences in available vegetation. Hana Hwy and Kipahulu sites were remote and surrounded by dense tropical forest with many natural oviposition sites. In contrast, Kaupo is a perennial grassland with very few natural oviposition sites. Sites that were proximal to human habitation included Hana Health, Kaumahina State Park, Keokea, Maui Invasive Species Council, Olowalu, Waihee, and Wakina. These sites were highly variable in available vegetation and elevation (Table 2.1). While these factors were not used in the analysis of *Ae. albopictus* oviposition, they may contribute to the spatiotemporal variation demonstrated.

Culex quinquefasciatus was present throughout the year (as evidenced by both eggs and adults) and was found across the island, however the numbers of adults captured was low, and abundance could not be statistically assessed. *Aedes vexans* and *W. mitchellii* were detected but seem to be less widely distributed or active throughout the year. The detection of these species may be seasonal and based on habitat *Ae. vexans* was only detected in April and June at site Kealia and Maui Forest Bird Recovery (Table 2.1). *Wyeomyia mitchellii* was detected in August, October, and February at sites Kealia, Maui Forest Bird Recovery and Maui Invasive Species Council. Limited collections of *Ae. vexans* and *W. mitchellii* were possibly due to a mismatch between sampling protocol and mosquito ecology.

Aedes vexans, is a floodwater mosquito that is typically detected during heavy rain periods (98). Ovipositing females lay their eggs in areas that are easily flooded (e.g. detention ponds, irrigated fields, and permanent water bodies where the water level fluctuates) and are not commonly found in artificial containers (99). Therefore, I would not expect them to be found in the oviposition traps used in this study. Nonetheless, adults were collected in this study. The collection of adults was limited to two sites: Kealia, which is a low elevation (20 m) site that is easily flooded during heavy rainfall, and Maui Forest Bird Recovery, which is a high elevation site (1153 m), that also receives heavy rainfall throughout the year. There have been verified sightings of *Ae. vexans* on Oahu at Puʻu ʻUalakaʻa State Park which is approximately 400 m in elevation (inaturalist.org). A study in Colorado also identified this mosquito species at ~1500 m elevations (98). Given that this species only hatches in certain conditions (i.e., floodwaters, or fluctuation water levels), it was not expected to capture this mosquito throughout the sampling periods (98, 99). Indeed, *Ae. vexans* was only capture during 2 of the 6 sampling periods.

Wyeomyia mitchellii larvae are typically found within in freshwater within bromeliads (100). Thus, again it was not unexpected that they were not detected with ovipositional traps used in this study. Adult *W. mitchellii* individuals were collected infrequently at low elevation sites (Kealia Pond) and high elevation sites that receive heavy rainfall (Maui Forest Bird Recovery and Maui Invasive Species Council). Bromeliads were noted at Maui Forest Bird Recovery and Maui Invasive Species Council, however to my knowledge, these were not present at Kealia Pond. Trap type and placement likely impacted the detection of *W. mitchellii*, my sampling efforts did not allow us to exclude the possibility that *Ae. vexans* and *W. mitchellii* was present in higher abundance and greater distributions.

Culex quinquefasciatus is a widely distributed vector of human and zoonotic pathogens and has been attributed to the autochthonous transmission of *Plasmodium relictum* in native Hawaiian honeycreepers (36). Generally, *Cx. quinquefasciatus* was detected in diverse habitats that varied in that vary in mean rainfall and temperature, and elevation (Table 2.1). For instance, the site Lahaina which captured majority of *Cx. quinquefasciatus* larvae (Table 2.3) is a warm low elevation site that is quite developed and in proximity to human habitation. Interestingly, wild chickens were observed in close proximity to the oviposition traps. The site at Maui Forest Bird Recovery captured the second highest amount of *Cx. quinquefasciatus* larvae and 4 adults. Interestingly, the *Ae. albopictus* larvae were not captured at site MFBR despite the presence of adults. The site Maui Forest Bird Recovery is high elevation, receives heavy rainfall, and is close proximity to human habitation with numerous avian host (Table 2.1). Four individuals were also collected at Hana Health, which was abundant in wild chickens and in close proximity to human habitation. Two individuals were captured at Olowalu and Kealia, which are both warm low elevation sites that are in proximity to human habitation and avian host (Table 2.1). Previous research has indicated that *Cx. quinquefasciatus* is restricted to elevations below 700 m (101), however this study and other studies have solidified that reproducing *Cx. quinquefasciatus* are found in high elevations (36, 101, 102). The highest elevation site in this study was 2051 m, however no mosquitoes were detected at this location. In the advent of climate change with temperatures increasing at higher elevations, future studies should incorporate multiple high elevation sites to assess *Cx. quinquefasciatus* expansion into new habitats, as this may have implications for avian malaria management. The ability of mosquitoes to reproduce in high elevations may shift in accordance with increasing global temperatures (7). Due to small number of adult mosquitoes captured, and infrequent detection at sampled locations, the influence of climate variables on the distribution of *Cx. quinquefasciatus* was not assessed, and my sampling efforts did not allow us to exclude the possibility that this mosquito species was present in higher abundance and greater distributions.

While only four mosquito species were captured, there have been historical sightings of *Ae. japonicus* and *Ae. aegypti* on the island of Maui. *Aedes japonicus* had a verified sighting in 2014 and 2015 at Makawao, and in 2016 at Keokea on Maui (iNaturalist.org). There is, however, no published data as to its seasonal abundance or distribution on the island though it is reported to be established. The Hawaiʻi Department of Health Vector Control Branch Division identified *Ae. aegypti* December 2017 at a port of entry on the island of Maui on one occasion and did not collect it again subsequently or in any other traps over a broader area. The question of why neither *Ae. japonicus* or *Ae. aegypti* were captured can be addressed by an exploration of the factors that influence the probability of detection. A longer trapping period will increase the probability of capture. In other studies oviposition traps are typically set in the field for 7 days, in this study the oviposition traps were set for 3 days and at only 18 locations. Likewise, the adults traps were only set for a 24-hour period once every two months at 9 locations. More frequent trapping might increase the probability of capture of uncommon or sparsely distributed mosquitoes. The type of trap used can that bias for and against the species captured. In this study two types of infusions for oviposition traps (fresh water and grass-infused) were used. These collected two mosquito species *Ae. albopictus* and *Cx. quinquefasciatus* but failed to detect *Ae. vexans* and *W. mitchellii* in this study. Recent studies have shown that infusions with specific types of bacteria and cell densities influenced the attraction of gravid *Ae. aegypti* and *Ae. albopictus* (103). Perhaps different water formulations would have attracted other species of mosquito. The BG-Sentinel trap used in this study are designed to capture mosquito species of the *Aedes* genera, although it is capable of attracting other species (104). The adult traps were able to capture greater overall diversity but failed to capture *Cx. quinquefasciatus* in areas that oviposition was detected (Olowalu). The BG-Sentinel traps also did not attract *Ae. japonicus* which suggest that they may not be the best trap for this *Aedes* species. Increasing the probability of detection requires the use of several types of traps aimed at aquatic and terrestrial life stages (104-106), and the ability to set traps for longer periods of time. Given the diverse ecological environments mosquitoes may have, it is likely that certain species remain undetected if only one type of mosquito trap is utilized. The probability of capture for uncommon, rare, or newly invasive species is further reduced if there are an insufficient number of the right kinds of traps. This is mostly likely the case for species such as *Ae. japonicus* that may have a very limited distribution and low abundance on Maui and *Ae. aegypti* that was not confirmed as established at the time of this study and is still under surveillance for biosecurity purposes as an invasive species.

Effective vector control strategies utilize integrated mosquito management to reduce mosquito populations and mitigate mosquito-borne disease transmission (41). Management programs incorporate a variety of methods such as: source reduction, control of immature mosquito stages (i.e., larvae and pupae), control of adult mosquitoes, surveillance, insecticide resistance monitoring, public education, and record keeping of mosquito locations (i.e., up to date distribution maps). Surveillance data provides health agencies with where and when mosquito vectors are detected. Comprehensive mosquito distribution maps document the range of vector distribution, and how distribution is influenced by local environmental conditions are fundamental to effective MDB prevention strategies and are crucial to any management program. Surveillance protocols in Hawai`i are currently monitoring for mosquito species at seaports and airports (pers comm., Hawaii Department of Health, Vector Control Branch). While these ongoing surveillance activities at priority locations is incredibly valuable, it is also extremely important to conduct island-wide surveillance studies to assess the distribution and abundance of medically important mosquitoes, especially in the advent of a mosquito-borne disease outbreak. This study provided an assessment of the distribution and abundance of mosquitoes on the island of Maui, and to my knowledge, is the first assessment performed on this island.

A limitation of this study was the low capture rate of some species of mosquitoes throughout the study period. Ideally, future studies will incorporate increased trapping frequency and density, multiple types of traps to describe the range and abundance of mosquitoes. More intensive data collection on the vegetation and complex site characteristics, could help provide better capability to describe drivers of overall mosquito distribution and abundance. Clarifying the occurrence and distribution of mosquito vectors is fundamental to reducing mosquito-borne disease transmission and while this study provides a baseline, the distributions and abundance of mosquito complexes can and do change over time and future surveys of not only Maui, but other islands in the Hawaiian archipelago should be conducted to protect people and native wildlife from mosquito vectored disease.

CHAPTER 3

THE AEDES ALBOPICTUS (DIPTERA: CULICIDAE) MICROBIOME VARIES SPATIALLY AND WITH ASCOGREGARINE INFECTION

ABSTRACT

The mosquito microbiome alters the physiological traits of medically important mosquitoes, which can scale to impact how mosquito populations sustain disease transmission. The mosquito microbiome varies significantly within individual mosquitoes and among populations, however the ecological and environmental factors that contribute to this variation are poorly understood. To further understand the factors that influence variation and diversity of the mosquito microbiome, I conducted a survey of the bacterial microbiome in the medically important mosquito, *Ae. albopictus*, on the high Pacific island of Maui, Hawai‘i. I detected three bacterial phyla, Proteobacteria, Acitinobacteria, and Firmicutes, and twelve bacterial families: Anaplasmataceae, Acetobacteraceae, Enterobacteriaceae, Burkholderiaceae, Xanthobacteraceae, Pseudomonadaceae, Streptomycetaceae, Staphylococcaceae, Xanthomonadaceae, Beijerinckiaceae, Rhizobiaceae, and Sphingomonadaceae respectively. The *Ae. albopictus* bacterial microbiota varied among geographic locations, but temperature and rainfall were uncorrelated with this spatial variation. Infection status with an ampicomplexan pathosymbiont *Ascogregarina taiwanensis* (Monocystis:Lankesteria) was significantly associated with the composition of the *Ae. albopictus* bacteriome. The bacteriomes of mosquitoes with an *A. taiwanensis* infection were more likely to include several bacterial symbionts, including the most abundant lineage of *Wolbachia* sp. (Rickettsiales: Ehrlichiaceae) Other symbionts like *Asaia* sp. (Rhodospirillales:Acetobacteraceae) and several Enterobacteriaceae lineages were less prevalent in *A. taiwanensis* infected mosquitoes. This highlights the possibility that inter- and intra-domain interactions may structure the *Ae. albopictus* microbiome.

INTRODUCTION

A symbiotic microbiome comprises communities of microorganisms that modulate their host's organismal function by influencing several physiological traits (107). Complex host-microbiome interactions can shape immunity (19), nutrition, and metabolism (81, 108); and alter host susceptibility to pathogens (26). For example, the symbionts *Hamiltonella defensa* (Enterobacterales: Enterobacteriaceae) and *Serratia symbiotica* (Enterobacterales:Yersiniaceae) have been shown to confer protection against parasitic wasps in aphids (109, 110), indicating a symbiont-mediated resistance against parasitism, and highlighting the intimate relationships between symbionts and their host that is shaped over evolutionary history.

In vectors, such as mosquitoes, microbiome modulation of host organismal function scales to influence the capacity of vector populations to transmit pathogens (111). Several vector traits that shape vectorial capacity are influenced directly and indirectly by the microbiome (112). For instance, certain microbial symbionts may enhance susceptibility of *Anopheles* sp. mosquitoes to *Plasmodium* sp. parasites, while endosymbionts like *Wolbachia* sp. decrease susceptibility of *Aedes* sp. and *Anopheles* sp. mosquitoes to arboviruses and *Plasmodium* sp. respectively (63, 113, 114). This change in vector competence might be associated with competition between microbial symbionts for resources, the release of anti-pathogen peptides that directly limit pathogen growth and extend the incubation period, or through the regulation of specific components of the insect immune system (111, 115).

The mosquito microbiome varies significantly between and within host species (64, 116). Mosquitoes acquire their microbial symbionts from the environment across all stages of development and via vertical transmission of endosymbionts early in embryogenesis (65). Several studies have demonstrated that the microbiome varies between individuals within a species depending on the host's sex, life stage, or geographic location (69, 117). Distinct habitats found across geographic space suggests that the environment might shape the mosquito microbiome, and that heterogeneity in the environment may result in the variation seen among mosquito populations (30), potentially contributing to spatial variation in mosquito-borne disease transmission.

Interactions between co-symbiotic microbes within a host might also shape the mosquito microbiome. For instance, recent studies have found that co-occurring bacteria were negatively correlated with other bacterial taxa in medically important mosquitoes (64). Specifically, *Wolbachia* sp. was found to be negatively correlated with bacterial taxa *Serratia* sp. and *Aeromonas* sp. (Aeromonadales: Aeromonadaceae), highlighting the complex interaction networks that may occur among symbionts in the mosquito host (64). Several *Aedes* sp. mosquitoes are host to an apicomplexan pathosymbiont, *Ascogregarina taiwanensis* (118). These parasites have a complex life cycle in their mosquito host that begins with the ingestion of oocysts that mature and release of sporozoites. These invade midgut epithelial cells and develop into trophozoites that migrate selectively to the Malpighian tubules. Here, they develop into macro- and microgametes that fuse to form diploid gametocysts, within which infectious oocysts mature (Figure 3.1) (119). Oocyst are eventually shed into the aquatic environment during the metamorphosis transition (pupae to adult), which will be ingested by subsequent 1st instar larvae that develop in the same aquatic environment. *A. taiwanensis* broadly infect mosquitoes (Diptera: Culicidae) and sand flies (Diptera: Psychodidae) and are globally distributed (119-121). The host specificity of *A. taiwanensis* remains unresolved. Some studies suggest a high host specificity (122), while others demonstrate contradictory results (123). Recent studies have found various dispersal strategies used by *A. taiwanensis* based on host sex and environmental cues (119). While Ascogregarine parasites in mosquitoes have served as a model system to investigate the evolutionary biology and physiological impacts of parasitism, their association with the composition of the mosquito microbiome and potential use as a biocontrol (120) remains poorly resolved.

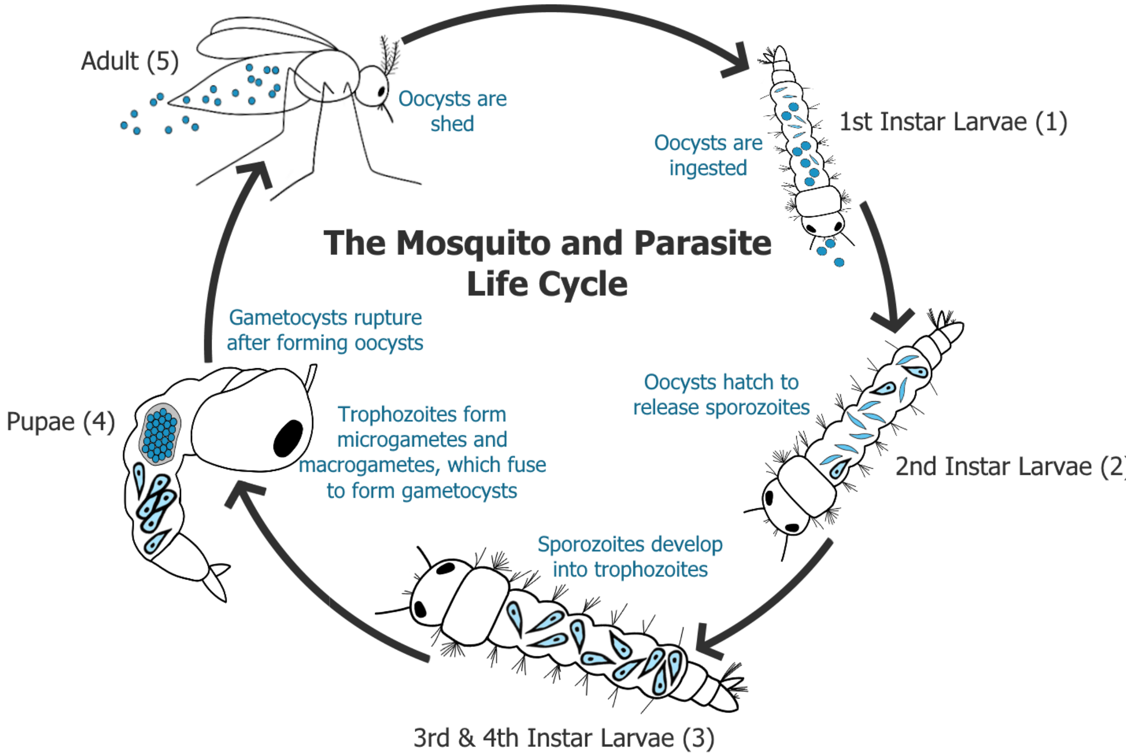

Figure 3.1: The *Ascogregarina taiwanensis* lifecycle. 1) Development of *A. taiwanensis* is initiated when oocysts are ingested from the aquatic environment by a 1st instar larva. 2) Oocysts release sporozoites into host epithelial cells. 3) Sporozoites develop into trophozoites. 4) Before pupation, trophozoites travel to the Malpighian tubules and develop into either micro- and macrogametes that may fuse to form gametocysts. Oocysts develop within the gametocyst. 5) Oocysts are eventually shed into aquatic environment during the transition from pupae to adult. Lifecycle adapted from Tseng, 2007 (124).

In this study, I investigated specific environmental and ecological factors that influence diversity in the bacteriome of the medically important *Aedes albopictus* mosquito. *Aedes albopictus* is a major vector of rapidly emerging chikungunya virus (125), and is widespread throughout Hawaiʻi (38). Mosquito samples were collected for an entire year across eight sites on Maui, HI. Maui was chosen because it is a high Pacific island (3,055 meters) that has drastic environmental gradients (126) in monthly average temperature (~8^{o}C to 32^{o}C) and rainfall (~0.25mm to 710mm) across relatively short geographical distances (5-60 km). As a result, high oceanic islands such as Maui, can serve as a natural model to assess how ecological and environmental factors influence the assembly and composition of the mosquito microbiome. My results suggest that the bacteriome of *Ae. albopictus* on Maui varies greatly between sites but is independent of the gradients in monthly average temperature and rainfall across these sites. In addition, I show that the composition of the *Ae. albopictus* microbiome is correlated with the host's intensity of infection with *A. taiwanensis*. The resulting data imply that cross-domain interactions in co-symbiosis may be a powerful modulator of the microbiome of a globally important disease vector.

METHODS

Mosquito collection

Adult female (n=96) and male (n=22) *Ae. albopictus* mosquitoes were collected at eight sites across Maui, HI at two-month intervals over the course of a single year. While collection occurred six times over the course of a year, mosquitoes that were suitable for analysis were only collected during five of the periods at eight sites during 2017. Mosquitoes were collected at each site using BG II Sentinel Traps (Biogents, Rogensburg, Germany), powered with Power-sonic batteries (San Diego, CA) and baited with CO_2 (Airgas, Hawai‘i) and BG lure (BioQuip, Rancho Dominguez, California).

A single BG II Sentinel trap, which tends to capture a majority of host-seeking females (127), was deployed for 24 hours at each site. After the 24 hour collection period, all mosquitoes were removed from the trap and identified to species, sex, and gravid status using a standard 10-40X dissecting scope and the Darsie and Ward taxonomic key (89). Gravid status was assessed based on visualizing the size of the abdomen. The number of *Ae. albopictus* collected varied across sites and sampling periods (Appendix F). Of the mosquitoes collected, only adults that were alive and intact at the time the trap was collected were used to characterize the microbiome. Blood-fed and gravid (i.e., enlarged abdomen) females were excluded from analysis. When possible, 1-10 individual mosquitoes (mean=2.95, median=2) were selected per site and sampling period and collection period and processed individually as samples for analysis (Appendix F). Once identified and selected for analysis, mosquito samples were stored in 70% ethanol and cooled on dry ice until they were returned to the laboratory and stored at -80^{o}C.

Average temperature (127) and rainfall (128) measurements were acquired from each site and collection period from the Climate Atlas of Hawaii (Appendix E). These sites ranged in altitude from 18 to 975 m and spanned monthly rainfall and temperature gradients of 2.29 to 271.4 mm and 15 to 26°C respectively (Appendix E).

<u>*Sample extraction and sequencing*</u>

Before extraction, samples were surface sterilized by one 70% ethanol wash, followed by two rinses in sterile 1X PBS, a protocol adapted from previous studies (129). DNA was then extracted and purified using a QIAamp DNA Mini Kit (Qiagen, Hilden, Germany) following the manufacturer’s protocol. Library preparation was done using a modified version of the Earth

Microbiome Project 16S V4 protocol (130). PCR amplification targeted the V4 region of bacterial 16S rRNA by using Earth Microbiome dual indexed barcoded primers. These primers included standard 515F (5'-GTGCCAGCMGCCGCGGTAA-3') and 806R (5'-GGACTACHVGGGTATCTAAT-3') 16S primers, along with unique attached barcodes (130). Each PCR reaction contained the following components: 16.25 µL of nuclease-free water, 5.0 µL of 5X KAPA HiFi Fidelity Buffer,1.0 µL of template DNA (approximately 10-87 ng), 0.75 µL of 10 mM KAPA dNTP Mix, 0.75 µL of 10 mM forward primer, 0.75 µL of 10 mM reverse primer, and 0.5 µL of 1 U/µL KAPA HiFI HotStart DNA Polymerase. A no-template PCR reaction was also performed as a negative reagent blank. PCR amplifications were performed in an Applied Biosystems SimpliAmp Thermal Cycler (Thermo Fisher Scientific, Waltham, MA) using the following conditions: initial denaturation at 95°C for 3 min; 35 cycles of denaturation at 98°C for 20 s, annealing at 60°C for 15 s, extension at 72°C for 30 s; and a final extension at 72°C for 30 s. The PCR products were visualized on a 2% agarose gel before being purified and normalized to approximately 1.25 to 2.50 ng/µL using a Just-a-Plate kit (Charm Biotech, Cape Giradeau, MO). Purity and concentration of a subset of samples were assessed with a NanoDrop (Thermo Fisher Scientific, Waltham, MA). A volume of 6 µL from each sample was pooled and then purified using a 1.2X volume of SerapureBeads (131). The library was checked for quality and quantity using a Bioanalyzer High Sensitivity chip (Agilent Technologies, Santa Clara, CA) run by Advanced Studies in Genomics, Proteomics and Bioinformatics at the University of Hawaiʻi at Mānoa. Once quality was confirmed, the library was sequenced (UC Davis Genome Center) using Illumina MiSeq PE300 with the MiSeq reagent kit v3 (Illumina, San Diego, CA).

Bioinformatics analysis

Raw paired fastq reads were preprocessed using the dada2 R package (25). Reads were filtered with the filterAndTrim() function. Reads were truncated at position 220 (190 for the reverse read) and discarded if they contained at least one base below quality 2 or a number of expected errors above 3. Denoising was performed using the learnError() and dada() functions with default parameters. Using the mergePairs() function, reads were merged if they overlapped by at least 20 bases, and a maximum of one mismatch was allowed. Mothur was used along with the Silva database (version 132) (downloaded from https://mothur.org/wiki/Silva_reference_files) to align and annotate the sequences. Sequences with a start or stop position outside the 5th - 95th percentile range (over all sequences) were discarded. Potential chimeras were removed with chimera.uchime(), and clustered at 99% similarity thresholds with chimera.vsearch(). Taxonomies were assigned using classify.seqs() and classify.otus(). The lulu R package was used to refine operating taxonomic units or OTUs (132). Two OTUs were merged if all of the three following conditions were satisfied if *i*) they co-occur in every sample, *ii*) one of the two OTUs has a lower relative abundance than the other in every sample, and *iii*) they share a sequence similarity of at least 99%. Finally, all singletons (OTUs with reads in only one sample) and OTUs with no annotation at the kingdom level were removed.

After quality control, sequencing resulted in 19.5 million reads (per sample mean: 79,864; per sample median: 76,085). OTUs that represented less than 0.01% of reads and had less than 5% prevalence were excluded from further analysis. Samples with less than 4,000 reads were also removed. OTUs found in the negative-reagent blank controls were also removed from analysis as they likely represent contaminants along the molecular analysis pipeline. These included the taxa *Delftia* sp., (Burkholderiales:Comamonadaceae), *Herbaspirillum* sp.

(Burkholderiales:Oxalobacteraceae) and Sandaracinaceae sp. Overall, 18 OTUs were used for downstream analysis, including two *Wolbachia* sp. strains (wAlbA and wAlbB), one *Asaia* sp., four bacteria from Enterobacteriaceae, and nine other taxa in the bacterial families *Burkholderiaceae*, *Xanthobacteraceae*, *Pseudomonadaceae*, *Streptomycetaceae*, *Staphylococcaceae*, *Xanthomonadaceae*, *Beijerinckiaceae*, *Rhizobiaceae*, and *Sphingomonadaceae*.

<u>*Estimation of Ascogregorina taiwanensis load*</u>

Total *Ascogregorina taiwanensis* infection intensity was assessed by qPCR using custom-designed primers (AT_SHORT_2_F: 5'-TCGATGAAGGACGCAGCTTA-3', AT_SHORT_2_R: 5'-AGGCACTGAACTGGACATACT-3') based off of an *A. taiwanensis* reference sequence (GenBank AY326461.1). Each qPCR reaction contained the following components: 5.0 µL of PowerUp SYBR Green Master Mix, 3.0 µL of nuclease-free water, 1.0 µL of template DNA (approximately 10-87 ng), 0.5 µL of 10 mM forward primer, and 0.5 µL of 10 mM reverse primer. The reactions were performed using an Applied Biosystems StepOnePlus Real-Time PCR System (Thermo Fisher Scientific, Waltham, MA) using the following conditions: UDG activation at 50°C for 2 min; Dual-Lock DNA polymerase at 95 °C for 2 min; 40 cycles of denaturation at 95°C for 15 s, annealing at 56°C for 15s, and extension at 72°C for 1 min. Fluorescence readings were taken at the 56°C annealing step for each cycle. A melt curve was performed according to PowerUp SYBR protocol to confirm the specificity of amplification. The Cycle threshold (Ct) values were used to measure *A. taiwanensis* infection intensity and were obtained assuming a delta Rn fluorescence threshold of 0.25. Samples were considered negative if the Ct value was greater than 38, or if the melt curve did not align with the positive control.

Estimation of Wolbachia load

Total *Wolbachia* sp. load was quantified through qPCR using *Wolbachia*-specific primers (*W*-Spec-16S-F: 5'-CATACCTATTCGAAGGGATA-3' and W-Spec-16S-R: 5'-AGCTTCGAGTGAAACCAATTC-3') primers (Werren and Windsor, 2000). Primers of the actin gene, alb-act-F (CCCA CACAGTCCCCATCTAC) and alb-act-F (CGAGTAGCCACGTTCAGTCA) were used to quantify host genomic copies. Each qPCR reaction contained the following components: 5.0 µL of 2X PowerUp SYBR Green Master Mix (Applied Biosystems), 1.0 µL of nuclease-free water, 1.0 µL of 10 µM forward primer, 1.0 µL of 10 µM reverse primer, and 2.0 µL of template DNA (approximately 10-87 ng). The reactions were performed using an Applied Biosystems StepOne Plus Real-Time PCR System (Thermo Fisher Scientific, Waltham, MA) using the following conditions: UDG activation at 50^{o}C for 2 min; Dual-Lock DNA polymerase at 95^{o}C for 2 min; and 40 cycles of denaturation at 95^{o}C for 15 s and annealing/extension at 60^{o}C for 1 min. Fluorescence readings were taken at the 60^{o}C annealing/extension step for each cycle. A melt curve stage was performed according to PowerUp SYBR protocol to confirm the specificity of amplification. The Cycle threshold (Ct) values were used to measure load intensity and were obtained assuming a delta Rn fluorescence threshold of 0.3. The relative abundance (i.e. *Wolbachia* index) was estimated as a ratio of the inverse Wolbachia Ct value to the inverse Ct of the single copy mosquito gene, actin. qPCRs with no detected Wolbachia template received a Ct value of 41 (total cycles in the reaction plus one).

Estimation of Asaia load

Total *Asaia* sp. load was quantified using custom-designed primers (ASAS_1_F: 5'-CGGCAACCTGGCTCATTAC-3', ASAS-1-R: 5'-ACATCCAGCACACATCGTTTAC-3') based off of a partial 16S rRNA sequence obtained from lab cultures isolated from *Ae. albopictus* midguts. Each qPCR reaction contained the following components: 5.0 µL of 2X PowerUp SYBR Green Master Mix (Applied Biosystems), 3.0 µL of nuclease-free water, 0.5 µL of 10 µM forward primer, 0.5 µL of 10 µM reverse primer, and 1.0 µL of template DNA (approximately 10-87 ng). The reactions were performed using an Applied Biosystems StepOne Plus Real-Time PCR System Thermo Fisher Scientific, Waltham, MA) using the following conditions: UDG activation at 50ºC for 2 min, Dual-Lock DNA polymerase at 95ºC for 2 min; 40 cycles of denaturation at 95ºC for 15 s, annealing at 58ºC for 15 s, and extension at 72ºC for 1 min. Fluorescence readings were taken at the 58ºC annealing step for each cycle. A melt curve stage was performed according to PowerUp SYBR protocol to confirm the specificity of amplification. The relative abundance (i.e. *Asaia* index) was estimated as a ratio of the inverse *Asaia* Ct value to the inverse Ct of the single copy mosquito gene, actin. qPCRs with no detected *Asaia* template received a Ct value of 41 (total cycles in the reaction plus one).

<u>*Statistical analysis*</u>

Generalized linear mixed models implemented in the package glmmTMB in program R were used to test a relationship between species richness (the number of bacterial OTUs per mosquito) and the fixed effects of rainfall, temperature, and *A. taiwanensis* infection. Linear mixed models implemented in package lme4 (133) in program R were used to test a relationship between logit transformed species evenness (the Simpson's index of each mosquito's microbiota, estimated with the function "diversity" in the package vegan) and the fixed effects of rainfall, temperature, and *A. taiwanensis* infection (133). Site and month were included as random effects in these models. Richness was measured as the total number of OTUs in a sample, and unbalanced read counts across samples was controlled for with the "offset" function in the base package of R. The significance of individual independent effects on α-diversity indices was assessed through a log-likelihood ratio test that compared a full model and a nested model that lacked an independent variable of interest. I assumed the log-likelihood approximates a Chi-square distribution.

I used zero-inflated generalized linear mixed models implemented in the package *glmmTMB* (134) in program R to test for differences in the composition of the microbiota from host mosquitoes across sites and month in relation to rainfall, temperature, mosquito sex and intensity of *A. taiwanensis* infection. The relative abundance of each bacterial taxon in each mosquito individual was calculated to create a single response variable. To accomplish this, the OTU community matrix was transformed from wide form (in which each row dictates an individual host mosquito coupled with columns populated with read counts of specific symbionts in that host) to long form (in which each row is a unique combination of an individual host and a bacterial taxon coupled with columns that show the read count of the bacteria in that host individual). As microbiome data is inherently composite in nature (149) and each read count for a given taxon should only be interpreted in the context of the total reads for that sample, the models were fitted assuming a binomial error distribution. Given that several observations (rows) in this dataset are tied to a specific symbiont taxon (in this data, there are 118 observations per individual bacterial taxon), symbiont taxa (18 levels in this dataset) were modeled as a random intercept in all candidate models. In addition, as the proportional data (reads from a specific bacterium of the total bacteria reads) were highly over-dispersed, an observation-level random intercept was fit to allow for overdispersion in all candidate models.

I use random slopes and random interactions with their corresponding fixed and main effects to allow the relative abundance of each OTU to independently change in response to explanatory variables in the model. The effect of monthly average rainfall, monthly average temperature, and *A. taiwanensis* infection intensity status on the microbiota composition were tested by modeling random slopes for each symbiont taxon, while site, month, and sex were tested as random interactions. All random slopes were modeled as uncorrelated with the random intercept. Zero-inflation was allowed to vary across the levels of the site × OTU taxa random interaction, and the random intercept within symbiont taxa in each model. The significance of these random slopes and interactions was assessed to test for changes in the composition of the microbiome using log-likelihood ratio tests. The conditional modes of these responses reveal effect sizes. In addition, random interactions of "site x Ascogregarina presence", "OTU x Ascogregarina presence x sex" and "OTU x Ascogregarina presence x site" were also tested to assess whether the effects of Ascogregarine infection were consistent across levels of "sex" and "site" (Appendix D).

In addition to the analyses above, I describe the data with the average proportion of reads for each OTU, which was calculated for individual mosquitoes at each sampling site. The OTU relative abundances of each symbiont were converted into presence-absence data for each site and the percentage of mosquitoes hosting each symbiont at each site were calculated. The presence-absence data for *A. taiwanensis* infection were used to calculate the prevalence of individual mosquitoes at each site that were positive for the non-metazoan parasite (Appendix I). I use the best-fit model to produce predicted probabilities for each symbiont taxa in a mosquito host with an intense Ascogregarine infection (i.e., the lowest Ct value in these data) and a host with no detectable Ascogregarine infection (i.e. no detected amplification with the qPCR protocol). I then use these predicted probabilities to estimate odds ratios to demonstrate the direction and effect size for each symbiont taxa. Mixed models implemented in the package *glmmTMB* in program R were also used to interrogate the probability of *A. taiwanensis* infection in relation to the fixed effects of rainfall, temperature, *Wolbachia* sp. index, *Asaia* sp. index, and random effects of site, month, and sex. Effects were tested with a log-likelihood ratio test, as previously described.

RESULTS

<u>*The Aedes albopictus bacteriome.*</u>

A total of 716 bacterial OTUs were identified from 118 adult *Ae. albopictus* mosquitoes across the island of Maui (Appendix E), 18 of which had a prevalence greater than 0.05% (Figure 3.2; Appendix G). Among the18 OTUs, three bacterial Phyla (Proteobacteria, Acitinobacteria, and Firmicutes) and twelve bacterial families were detected: *Anaplasmataceae*, *Acetobacteraceae*, *Enterobacteriaceae*, *Burkholderiaceae*, *Xanthobacteraceae*, *Pseudomonadaceae*, *Streptomycetaceae*, *Staphylococcaceae*, *Xanthomonadaceae*, *Beijerinckiaceae*, *Rhizobiaceae*, and *Sphingomonadaceae* (Figure 3.2). Within the bacterial family *Anaplasmataceae*, two *Wolbachia* sp. lineages, *Wolbachia sp.* wAlbB, and *Wolbachia sp.* wAlbA, accounted for 89% of the sequencing reads, and were found in 99% of samples. The symbiont within the genus *Asaia* of the Acetobacteriaciae family accounted for 3.9% of overall sequencing reads and was found in 30% of samples. Several other OTUs belonged to the family Enterobacteriaceae. With the exception of a *Serratia marcescens* (Enterobacterales: Yersiniaceae). (7% of samples, 1.7% sequencing reads), the genus of these Enterobacteriaceae taxa could not be determined conclusively. These taxa were labelled Enterobacteriaceae 1-4. The most supported taxonomic identity (as indicated by the Basic Local Alignment Search Tool on GenBank) is as follows: Enterobacteriaceae 1 (likely *Klebsiella* <u>sp.</u> or *Enterobacter* <u>sp.</u>, 13% of samples, 1.5% sequencing reads), Enterobacteriaceae 2 (likely *Pantoea* <u>sp.</u>, 10% of samples, 1.5% sequencing reads), Enterobacteriaceae 3 (likely *Cedecea* sp. or *Klebsiella* sp., 11% of samples, 0.97% sequencing reads), and Enterobacteriaceae 4 (likely *Yersinia* sp. or *Serratia* sp., 22% of samples, 0.05% sequencing reads). Bacterial genera *Burkholderia* sp. (Burkholderiales: Burkholderiaceae) was detected in 25% of samples and 0.26% of sequencing reads. Other OTUs were detected in the samples at varying levels, such as a Rhizobiales (9% of samples, 0.07% sequencing reads), *Bradyrhizobium* (Hyphomicrobiales:Nitrobacteraceae) (14% of samples, 0.05% sequencing reads), *Pseudomonas* sp. (Pseudomonadales:Pseudomonadaceae) (9% of samples, 0.05% sequencing reads), *Streptomyces* sp. (Streptomycetales: Streptomycetaceae) (13% of samples, 0.04% sequencing reads), *Staphylococcus* sp. (Bacillales:Staphylococcaceae) (5% of samples, 0.03% sequencing reads), *Strenotrophomonas* sp. (Xanthomonadales:Xanthomonadaceae) (11% of samples, 0.02% sequencing reads), *Methylobaterium* sp. (Hyphomicrobiales: Methylobacteriaceae) (7% of samples, 0.02% sequencing reads), Rhizobium (10% of samples, 0.008% sequencing reads), and *Spingomonas* sp. (Sphingomonadales:Sphingomonadaceae) (7% of samples, 0.01% sequencing reads). The OTUs Rhizobiales and Rhizobium could not be classified to lower taxonomic rankings.

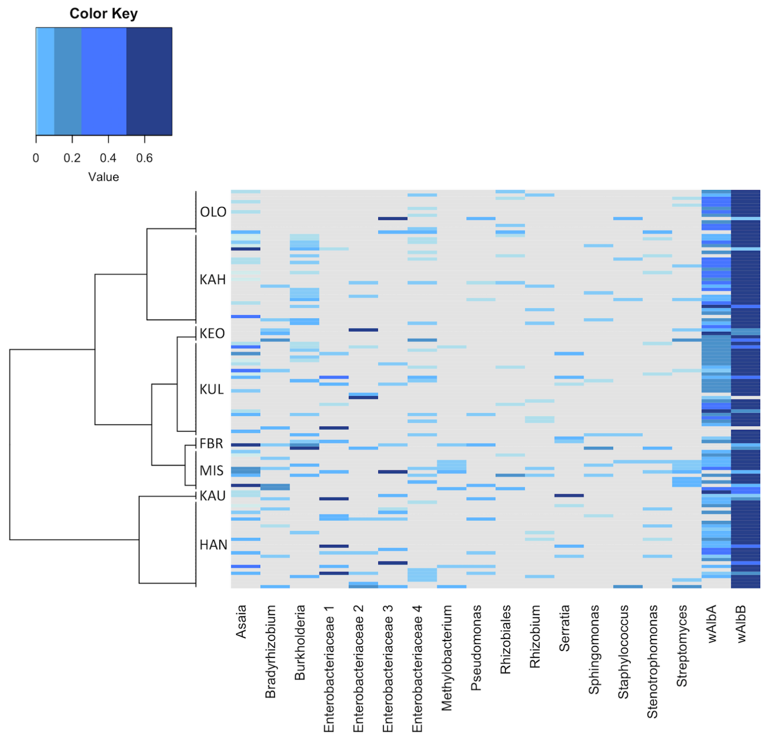

Figure 3.2. Heatmap showing the prevalence of bacterial taxa within an individual mosquito distributed across sites. Cell values are calculated as proportions across rows. The site dendrogram estimated with Euclidean distances based on geo-spatial data. Site's abbreviations; Forest Bird Recovery (FBR), Hana (HAN), Kahului (KAH), Kaupo (KAU), Keokea (KEO), Kula (KUL), Maui Invasive Species Council (MIS), Olowalu (OLO). Enterobacteriaceae taxonomic names had multiple hits when blasted against NCBI GenBank database and have been given family names for clarity. Enterobacteriaceae 1-4 were most similar to sequences identified as Klebsiella sp. and Enterobacter sp., Pantoea sp., Cedecea sp. or Klebsiella sp., and Yersinia or Serratia, respectively.

Effects on α -diversity of the Aedes albopictus microbiome.

When the *Ae. albopictus* microbiome was defined as a set of the 18 most prevalent bacterial taxa, the average (± standard error of the mean) species richness was 3.9 ± 0.14 and evenness (Simpson's index) was 0.30 ± 0.016. Temperature (p-value=0.37), rainfall (p-value=0.15), and *A. taiwanensis* infection (p-value=0.09) were not significantly associated with the richness of mosquito microbiotas (generalized negative binomial mixed model, Table 3.1). However, richness varied significantly across geography (site: p-value=0.016), and marginally insignificantly with time (month: p-value=0.074).

Table 3.1: The estimates and standard errors for variables included in the full linear mixed model to explain variation in species richness.

Fixed Effects	Estimate	Standard error
Intercept	-8.4	1.52
Temperature	-0.059	0.064
Rainfall	0.0056	0.037
A. taiwanensis	0.022	0.013
Conditional Model		
Random Effects	Variance	Standard Deviation
Site	0.17	0.42
Month	0.08	0.29

* The species richness was used as response variables in the linear mixed model.

Temperature (p-value=0.45), rainfall (p-value=0.47), and *A. taiwanensis* infection level (p-value=0.66) were not associated with rarefied evenness (Table 3.2). The random effects of site (p-value=1) and month (p-value=0.82) were also not associated with rarefied evenness, offering no support that rarefied evenness varied with any variable included in the analysis.

Table 3.2: The estimates and standard errors for variables included in the full linear mixed model to explain variation in species evenness.

Full Model	Estimate	Standard Error
Intercept	-0.29	1.5
Temperature	-0.048	0.063
Rainfall	-0.0018	0.0038
A. taiwanensis	0.013	0.019
Conditional Model		
Random Effects	Variance	Standard Deviation
Site	0.12	0.35
Month	0	0

Modeling β-diversity of the Ae. albopictus microbiome.

The first stage of this modelling effort tested spatiotemporal variation in the composition of the microbiome, while controlling for variables that vary at the level of individual mosquito hosts (i.e. Ascogregarine infection and host sex). The *Ae. albopi*ctus microbiome varied across sites (Figure 3.3 and Table 3.3; random interaction between site and OTU, p-value=0.0015). However, the effect of month was not significantly associated with variation in the composition of the mosquito microbiome (p-value=0.81). *Wolbachia* species *wAlbB* and *wAlbA* were the symbionts with the greatest relative abundance (89% of reads). *Wolbachia* sp. were found at all sites across Maui with their presence in mosquitoes varying from 50-100% across sampled sites (Appendix H). *Asaia* sp. was the next most prevalent species, found in all locations except Keokea with an average percentage of reads (0.021-21%) (Appendix G) and prevalence of 0-66% across sites (Appendix H). *Serratia* sp. prevalence varied from 0-33%, and was found in 50% of the sites sampled, with Kaupo containing the highest proportion of reads (Appendix G and H). Other environmentally acquired symbionts like Enterobacteriaceae 1-3 were detected across 0-33% of sites (Appendix H). The highest proportion of reads of Enterobacteriaceae 1 were detected in Kaupo (25%), while Keokea and Olowalu contained the highest proportions of Enterobacteriaceae 2 (14%) and Enterobacteriaceae 3 (7.8%) respectively (Appendix G, S4). The prevalence of the other symbionts varied across site from 0 to 50% (Appendix G, S4).

In the second stage of this modeling effort, environmental variables (temperature and rainfall) were tested as potential drivers of the significant spatial variation seen in the microbiome, while controlling for the same variables that vary at the level of individual hosts. Although rainfall and temperature varied across sites and over sampling periods (Appendix E), these environmental variables were not associated with composition of the microbiome in these data (rainfall p-value = 1.0, temperature p-value = 0.94).

Lastly, the individual level effects of Ascogregarine infection and host sex were tested as potential drivers of microbiome variation, while controlling for spatial variation. The composition of the microbiome was significantly associated with *A. taiwanensis* infection levels (Table 3.3; p-value=0.01). The probability of sampling *wAlbB* increased by a factor of 3.2 under intense *A. taiwanensis* infection relative to hosts with no detectable infection, while the probability of sampling *wAlbA* remained relatively unchanged (Table 3.4). Similarly, the odds ratios indicated that the probability of sampling Enterobacteriaceae 2, dramatically increased 7.5 times in the presence of an intense *A. taiwanensis* infection (Table 3.4). This was not consistent across the family, however, as Enterobacteriaceae 3 (OR=1.1) had a relatively modest increase and Enterobacteriaceae 1 (OR=0.06) and *S. marcescens* (OR=0.1) decreased in the presence of *A. taiwanensis* (Table 3.4). Other groups, such as *Asaia* (OR=0.41), and Bradyrhizobium (OR=0.26) also had a lower probability of being detected in mosquitoes with *A. taiwanensis* infection (Figure 3.4, Table 3.4). The odds ratios of other symbionts ranged between 0.05-1.5, suggesting that they did not change substantially in the host's microbiome across Ascogregarine infection intensity (Figure 3.4, Table 3.4). Finally, there was no relationship between sex of the mosquito and the microbiome composition (p-value = 0.37).

Table 3.3: The estimates and associated standard errors of fixed effects and the variance and associated standard deviation of the random effects in a mixed effects model that only included significant effects in the analysis interrogates the change in the composition of the microbiome within *Ae. albopictus* mosquitoes.

Full Model	Estimate	Standard error
Intercept	-4.7	0.50
A. taiwanensis	0.13	0.20
Conditional Model		
Random Effects	Variance	Standard Deviation
Site	0.16	0.40
OTU	3.5	1.9
Site x OTU	0.97	0.99
A. taiwanensis	0.35	0.59

* Best fit model included a row ID variable row ID to control for overdispersion and fixed effect for *A. taiwanensis* infection intensity.

Table 3.4: Odds ratios indicating predicted probabilities of sampling bacterial taxa when mosquitoes are highly infected with *A. taiwanensis* or not infected.

	Predicted probability from best fit model		
	High intensity Infection	No detectable Infection	Odds ratio
wAlbB	0.71	0.44	3.2
wAlbA	0.13	0.12	1.1
Asaia	$3.3x10^{-3}$	$8.0x10^{-3}$	0.41
Serratia	$8.1x10^{-4}$	$8.7x10^{-3}$	0.1
Enterobacteriaceae 1	$2.7x10^{-3}$	$4.1x10^{-2}$	0.06
Enterobacteriaceae 2	$8.8x10^{-3}$	$1.2x10^{-3}$	7.5
Enterobacteriaceae 3	$6.7x10^{-3}$	$6.0x10^{-3}$	1.1
Burkholderia	$5.1x10^{-3}$	$5.5x10^{-3}$	0.93
Enterobacteriaceae 4	$8.5x10^{-4}$	$3.4x10^{-3}$	0.25
Rhizobiales	$6.9x10^{-4}$	$8.6x10^{-4}$	0.80
Bradyrhizobium	1.7x10-3	$6.6x10^{-3}$	0.26
Pseudomonas	$1.0x10^{-3}$	$1.6x10^{-3}$	0.65
Streptomyces	$1.3x10^{-3}$	$4.0x10^{-3}$	0.32
Staphylococcus	$9.5x10^{-4}$	$8.8x10^{-4}$	1.1
Stenotrophomonas	$5.4x10^{-4}$	$4.3x10^{-4}$	1.3
Methylobacterium	$1.4x10^{-3}$	$5.4x10^{-4}$	2.6
Rhizobium	$3.1x10^{-4}$	$5.6x10^{-4}$	0.55
Sphingomonas	$5.1x10^{-4}$	$6.1x10^{-4}$	0.84

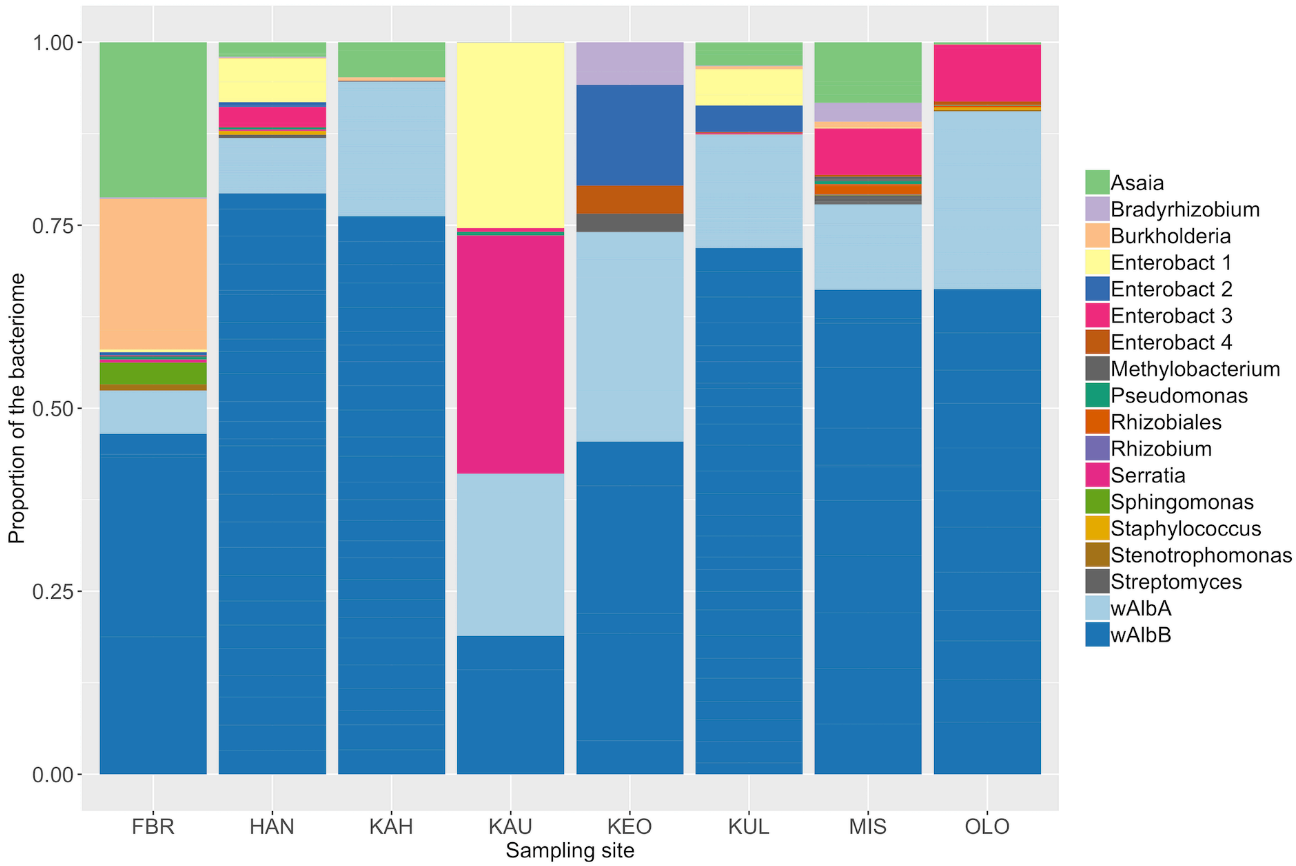

Figure 3.3: The composition of the bacteriome of *Ae. albopictus* among different sampling sites on Maui, HI. Figure represents bacteriome of mosquitoes collected over entire study (n=118): FBR (n=4), HAN (n=26), KAH (n=27), KAU (n=3), KEO (n=5), KUL (n=28), MIS (n=12), OLO (n=13).

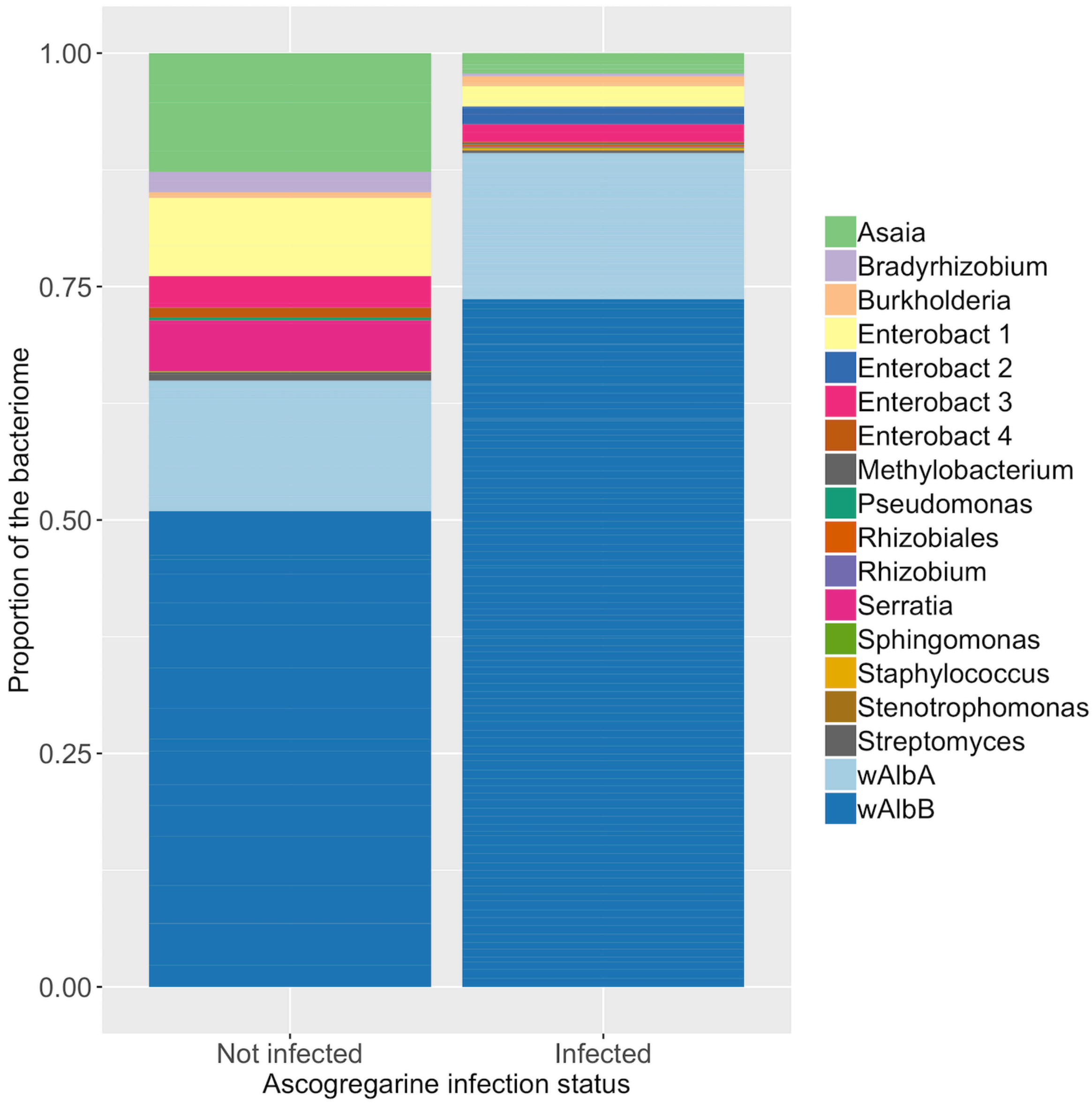

Figure 3.4: The composition of the *Ae. albopictus* bacteriome between *A. taiwanensis* infected (n=99) and uninfected (n=19) mosquito individuals.

Drivers of Ascogregarina infection

Shifts in the relative abundance of a focal symbiont (e.g. wAlbB) may be due to a change in the absolute abundance of that taxon, or the absolute abundance of the other taxa in the microbiome. To clarify the observation that the wAlbB and several environmentally acquired bacteria (e.g. *Asaia* sp.) change in relative abundance with the mosquito microbiome with *A. taiwanensis* infections status, I tested whether *A. taiwanensis* infections is correlated with an index of absolute abundance of *Wolbachia* sp. and *Asaia* sp. bacteria, while controlling for other environmental factors that might influence infection. Temperature (p-value=0.96), rainfall (p-value=0.65), and the index of *Wolbachia* sp. absolute abundance (p-value=0.60) were not significantly associated with the probability of *A. taiwanensis* infection (Table 3.5). However, there was a marginally significant association for the index of *Asaia* sp. absolute abundance (p-value=0.077), suggesting low *Asaia* sp. abundance in Ascogregarine infected mosquitoes. For example, mosquitoes with an *Asaia* sp. index of zero (i.e., no detectable *Asaia* infection) were 8.3X more likely to harbor an *A. taiwanensis* infection than mosquitoes that had an *Asaia* index of 1. The range of the *Asaia* sp. index in these data was 0.50-1.51, so an increase of 1 roughly corresponds to the change in *Asaia* sp. abundance from the least to the greatest values in the sample. The random intercept of site (p-value= 1), month (p-value=0.50) and sex (p-value=1) were not associated with *A. taiwanensis* presence, suggesting that the prevalence of *A. taiwanensis* is similar among collection sites, across months, and between the sexes (Table 3.5).

Table 3.5: The estimates and standard errors of fixed effects and the variances and standard deviation of the random effects in a mixed effect model that interrogates the change in *A. taiwanensis* infection between mosquitoes.

Full Model	Estimate	Standard error
Intercept	2.9	3.4
Temperature	0.0059	0.14
Rainfall	-0.0036	0.0077
Wolbachia sp. index	0.73	1.4
Asaia sp. index	-2.6	1.5
Conditional Model		
Random Effects	Variance	Standard Deviation
Site	0.43	0.65
Month	1.01×10^{-9}	3.2×10^{-5}

DISCUSSION

The microbiome of a mosquito alters the physiological traits of vectors that can scale to impact its vector capacity (30, 73). While many studies have shown that the microbiome varies within and between mosquito species (16), the specific factors that contribute to this variation are poorly resolved. The aim of this study was to understand how certain biotic and abiotic factors influence the composition of the microbiome of *Ae. albopictus*, a globally important vector of chikungunya virus (135). Broadly, the data suggest that the composition of *Ae. albopictus* bacteriomes on Maui are associated with a eukaryotic and parasitic co-symbiont, *Ascogregarina taiwanensis*. These microbiotas also change in composition across sites, but these changes are independent of stark environmental gradients of temperature and precipitation (Appendix E).

Previous studies have described interactions between co-symbionts within a mosquito host in structuring the overall host's microbiota (27, 136). For instance, the bacterial genera *Asaia* sp. may act antagonistically to impede the vertical transmission of *Wolbachia* sp. in *Anopheles* sp. mosquitoes (19), and is generally negatively correlated with the presence of *Wolbachia* sp. endosymbionts. Other studies extend these relationships to interdomain interactions. For instance, *Serratia marcescens* strains and *Chromobacteriaum Csp_P* impact the development of *Plasmodium* sp. in *Anopheles* sp. mosquitoes (137). My data suggests that the level of infection with another ampicomplexan parasite *A. taiwanensis* is significantly associated with the bacteriome of *Ae. albopictus*. Mosquitoes with *A. taiwanensis* infection were more likely to contain several bacterial symbionts including *wAlbB*, an Enterobacteriaceae bacteria that was most similar to *Pantoea* sp. and *Burkholderia* sp. Mosquitoes without an *A. taiwanensis* infection, were more likely to contain other symbionts, including *Asaia* sp., *Serratia marcescens,* and another Enterobacteriaceae bacteria that was most similar to *Klebsiella* sp. The results indicate that while a dramatic increase in relative abundance of *wAlbB* in *A. taiwanensis* infected mosquitoes was observed, the total abundance of *wAlbA and wAlbB* (i.e., *Wolbachi*a index) remained unchanged. This might be associated with shifts in the relative abundance of *wAlbA* relative to *wAlbB*. between *A. taiwanensis* infected and uninfected mosquitoes. Alternately, the apparent increase in relative abundance may also be due to a reduction in the abundance of environmentally acquired symbionts that compete for reads with *Wolbachia* sp. Indeed, mosquitoes that lacked an *A. taiwanensis* infections had an increase in the index of absolute *Asaia* sp. abundance. Although this effect was marginally insignificant, the trend of greater *Asaia* sp. absolute abundance in mosquitoes without *A. taiwanensis* infection comports with a hypothesis that the increase in apparent relative abundance of *Wolbachia* sp. is actually associated with a decrease abundance in some environmentally acquired bacterial symbionts. Further work (see below) is necessary explore this relationship.

The co-occurrence of *A. taiwanensis* life-cycle stages with environmentally acquired symbionts like *Asaia* sp. in specific organs and tissues provides ample opportunity for both direct and indirect interactions between these bacterial and eukaryotic symbionts. The infection of *A.*

taiwanensis occurs in the early aquatic life stages of mosquitoes (118) and thus proceeds through several tissues in the mosquito that are also important for the persistence and transstadial transmission of environmentally acquired bacterial symbionts, and may explain the compositional changes in the environmentally acquired bacterial microbiome (69, 138). For instance, the initial site of *A. taiwanensis* infection is the mosquito midgut, which harbors a diverse assemblage of symbionts and is a major site of primary colonization of environmentally acquired bacteria in the mosquito (111). In addition, the Malpighian tubules, which harbor *A. taiwanensis*, are conserved during metamorphosis at the pupal stage and have been implicated in the transstadial transmission of symbionts acquired during the larval stage to the adult stage, where disease transmission occurs (139).

Cumulatively, these data highlight the potential for interdomain interactions that may contribute to the diversity of the *Ae. albopictus* microbiome. Multiple mechanistic hypotheses might explain the apparent inter- and intra-domain associations identified in this study. Previous studies suggest that *Wolbachia* are associated in numerous interactions with other symbionts (64), either directly through co-exclusion with other bacterial symbionts or indirectly via immune activation pathways. Since *Wolbachia* sps. colonize their host during embryogenesis, these results might imply that *Wolbachia* facilitate *A. taiwanensis* infection. However, the similar absolute abundance of *Wolbachia* sp. between Ascogregarine-infected and non-infected mosquito host do not support this hypothesis. Conversely, *A. taiwanensis* infection may inhibit environmentally acquired symbionts, either directly via mechanical disruption of resident bacterial communities; or indirectly via modulation of immune pathways such as the immune deficiency (IMD) and Toll pathways (24). For example, the response to *A. taiwanenesis* infection might potentially induce the release of antimicrobial peptides (AMP) or inhibit C-type lectins (mosGCTLs) that results in the reduction of environmentally acquired symbiont colonization (24). Direct or indirect mechanisms likely have cascading effects that influence the overall composition of the *Ae. albopictus* microbiome and should be addressed in future studies. Ultimately, a controlled fully factorial experiment that manipulates infection with *A. taiwanensis*, *Wolbachia* sp., and other environmentally acquired bacterial symbionts would further characterize the direct and indirect interactions between co-occurring taxa and might resolve the ecological mechanisms by which inter- and intra-domain interactions contribute to the diversity in the microbiome of medically important mosquito vectors.

Several studies have shown that the ecological habitat in which mosquitoes develop may impact the composition of their microbiome (67, 116). For instance, *Culex nigripalpus* mosquitoes have been shown to display distinct microbiome compositions based on geographic locations (117). Similarly, my data suggest that bacterial microbiota of *Ae. albopictus* varies among sampling locations. However, despite stark environmental gradients in temperature and rainfall that varied spatiotemporally (Appendix E), these environmental variables were uncorrelated with changes in the composition of the *Ae. albopictus* bacteriome in this study. I originally hypothesized that distinct habitats and environments would create distinct source species pools of symbionts that are available to colonize mosquitoes, and that this would contribute to variation in the mosquito microbiome across space. A lack of correlation between temperature and rainfall, and the composition of the mosquito microbiome is surprising since both these environmental variables typically have strong impacts on mosquitoes directly, and structure the broad ecological communities that include local mosquito populations (29, 140). Furthermore, temperature itself strongly influences bacteria replication rates (141). Other environmental variables such as land use could play a stronger role in the heterogeneity seen in microbiomes. For example, land use practices are expected to affect biogeochemical cycling and microbiome compositions of soil (142). Similarly, these types of effects might modulate the microbiomes of different mosquito populations in urban, rural, agricultural, and natural environments, where land use and management practices vary. Alternatively, the variation in the mosquito microbiome across space might also be due to ecological drift that results from dispersal limitation and weak niche effects. Additional studies across a larger and finer spatial scale should explore how environmental variables like temperature, elevation, rainfall, land-use, and dispersal limitation might contribute to variation in the mosquito microbiome. The elevated islands of Hawaiʻi, with their large environmental gradients over short geographic scales and changing topography, remain a particularly valuable natural setting for these types of studies.

This study has identified two important factors that influence the *Ae. albopictus* microbiome: geographic location and Ascogregarine infection. I recognize that a limitation of this study was the relatively low number of mosquitoes per geographic location and sampling events across space and time. Ideally future studies would include higher sampling per geographic location and additional geographic sites to further assess explicit drivers of mosquito microbiome assembly. Clarifying which explicit environmental and ecological characteristics of geographic location that impact the structure and function of the mosquito microbiome is an essential area for future research and will inform my understanding of how vectorial capacity changes across landscapes. This study, and future studies with a similar focus, may shed light on niche effects that drive the assembly of the vector microbiome, perhaps enhancing the ability to predict vector-borne disease outbreaks. In addition, understanding the role of niche effects (165) and dispersal limitation (143) in structuring mosquito microbiotas could inform the development of future paratransgenic strategies that rely on modified bacteria that suppress pathogen carriage or transmission by a vector (144). Indeed, understanding complex microbial interactions is essential for developing a successful and stable paratransgenic platform (145). For instance, strong niche effects that modulate bacterial symbiont fitness in its host are expected to limit the distribution of the symbionts among mosquito vectors in a complex landscape. Cumulatively, understanding the explicit drivers of the mosquito microbiome assembly may have important implications in lowering the global burden of mosquito-borne disease.

CHAPTER 4

THE *AEDES ALBOPICTUS* MICROBIOME DERIVES FROM ENVIRONMENTAL SOURCES AND PARTITIONS ACROSS DISTINCT HOST TISSUES.

ABSTRACT

The mosquito microbiome consists of a consortium of interacting microorganisms that reside on and within the host. Mosquitoes likely acquire the majority of their microbial diversity from the environment during development. Once present within the mosquito host, the microbes disseminate across distinct tissues and symbiotic relationships are maintained via immune strategies, environmental filtering, and trait selection. The processes that govern how environmental microbes assemble across the tissues within mosquitoes remain unresolved. Here, ecological network analyses are used to examine how environmental bacteria assemble to form bacteriomes among *Aedes albopictus* host tissues (crop, midgut, Malpighian tubules, and ovaries). Mosquitoes, water, soil, and plant samples were collected from 20 sites along a 4 km transect along Mānoa Valley, Oahu. Results indicate that the bacteriomes of *Ae. albopictus* tissues were compositionally nested within the environmental bacteriome and suggest that the environmental microbiome likely serves as a source pool for mosquito-associated bacterial symbionts. Within the mosquito, the microbiomes of the crop, midgut, Malpighian tubules, and ovaries differed in composition. Subsets of this microbial diversity formed two specialized modules, one in the crop and midgut and another in the Malpighian tubules and ovaries. The specialized modules may form based on microbe niche preferences and/or selection of mosquito tissues on specific microbes that may aid unique biological functions of the tissue types. Strong niche-driven assembly of tissue-specific microbiotas from the environmental species pool suggests that each tissue has specialized associations with microbes, which perhaps derive from host-mediated microbe selection.

INTRODUCTION

Microbiomes are diverse ecological communities comprised of fungi, viruses, archaea, bacteria, and protozoans that often provide essential functions to their metazoan hosts (28). These functions provided to the host may change in response to turnover in microbiome composition. Indeed, microbiome diversity has been attributed to many phenotypes that underlie overall host function, including nutrient provisioning (17), metabolism (145), and protection from natural enemies (3, 19, 26). In mosquitoes, the microbiome is a major modulator of host nutrition, metabolism, reproduction, development, immunocompetence, and behavior (30, 72), and these effects can scale to impact the capacity of mosquito vectors to transmit infectious disease (146).

A primary feature of these symbiotic communities is substantial ecological diversity, including a large number of symbiont taxa in a microbiome assemblage and high turnover of these taxa among individual host microbiome assemblages(17, 146). One of the pressing challenges of microbiome science is understanding the drivers of this variation in nature (147). Current attempts to understand microbiome diversity have largely focused on individual host traits (143). For instance, a recent study that surveyed 1000 human individuals and measured approximately 500 individual host factors could only explain about 17% of microbiome variation found in the subjects (148). Despite the large amounts of metadata on hosts, the individual level host effects were not sufficient in explaining the majority of microbiome variation observed in the study.

The microbiome of mosquitoes consists of diverse populations of interacting symbiotic microorganisms that reside in mosquito hosts and may distribute differentially across mosquito tissues. Previous studies have demonstrated high diversity in the structure and composition of mosquito-associated microbial communities among mosquito species (16, 72), geographic sites (129), developmental life stages (72), host sex (72), and host tissue (149). Except for endosymbiotic *Wolbachia* sp., which are acquired early on in embryogenesis, mosquitoes likely acquire the majority of this diversity from their environment (69, 150). However, the environmental sources of these facultative symbionts and how they assemble within the mosquito hosts remain poorly resolved.

Mosquitoes interact with different microbial-rich environmental substrates at different stages of their amphibious life cycle. Research studies have shown that mosquito larvae have similar microbiome composition to their aquatic habitats (151). During the terrestrial adult life stage, a portion of the gut bacteria of mosquitoes such as *Asaia* sp. are shared with the microbiomes that form on sugary plant nectars (61). Indeed, removing mosquitoes from natural settings and allowing their microbiome to recruit in more sterile lab settings is associated with dramatic and consistent shifts in the mosquito microbiome (152). Broadly, these examples suggest that the environment plays a role in structuring mosquito microbiotas and may be the ultimate source of its diversity (68).

As microorganisms from the environment colonize mosquitoes, they also sort differentially among organs and tissue types. The mechanisms and consequences of this assembly process remain under described in mosquitoes. However, research in other invertebrate systems indicate that such differentiation might be related to niche effects associated with the microbe in different environments within the host as well as host-mediated microbe selection during the formation of the symbiosis. For example, *Euprymna scolopes* (Sepiida:Sepiolidae), is exposed to millions of bacteria in seawater, yet only a single strain of *Vibrio fisheri* (Vibrionales:Vibrionaceae) colonizes the light organ (153). Previous studies have demonstrated that the host sets up a series of chemical and physical barriers to microbial colonization that selectively promote the assembly of *V. fisheri* and inhibit colonization of potential microbial competitors in the light organ environment. Within the light organ, *V. fisheri* bioluminesces and reduces host mortality associated with predation through countershading in the water column. This system indicates that invertebrate hosts may actively maintain symbiosis, and select for microbes that provide a beneficial function (154).

Here, I use a series of ecological network analyses, including nestedness (environments that have low species richness subset from those with higher species richness (185)), modularity (a network in which compartments of interacting species form, and there is little to no interactions between species outside each compartment (155, 156), and specialization (species have little to no interaction partners (157) to determine the extent to which microbes are shared among tissues and environmental substrates that the mosquito interacts with over its life cycle. Specifically, I test a series of hypotheses that purport how microbes source and disseminate from the environment across distinct tissues of the host. I expect that if the environment is a source pool for potentially colonizing mosquito symbionts, then the mosquito microbiotas will be a subset from the environmental microbiome. Additionally, I expect that if mosquito host maintain their microbiome, then the microbiotas in tissues will form differentially. Clarifying aspects of the environment that contribute to mosquito microbiomes will shed light on the origin of microbial symbionts and their process of assembly to form a host-associated microbiome and can be used to inform vector control strategies such as paratransgenesis.

METHOD

Sampling Sites

Adult *Ae. albopictus* mosquitoes and environmental samples were collected at twenty sites within two main areas (UH Manoa and Lyon Arboretum) in Mānoa Valley on Oahu over a 6-month period in 2019. Sites were placed at a minimum 30 meters apart from each other. The sites were chosen as they represent a typical urban to natural environment mixture in Hawaii (Appendix J). For instance, Sites 1-9, 15 and 20 are proximal to human habitation with natural and artificial oviposition sites detected (Appendix J). Sites 10-14 and 16-19 had varied distance to human habitation which ranged from 5-15 meters. Sites 10-12 were located along a frequently visited trail, while sites 13,14, and 16-19 were approximately 30 meters from the trail head with reduced likelihood of encounter with people. Samples were collected on a single occasion at each site between June 2019- December 2019 (Appendix J). Seasonal variation was considered by inclusion of a random interaction between Site and OTU (described below in statistical analysis).

Mosquito Collection and Tissue Sampling

Mosquitoes were collected using an aspirator and were identified to species using the Darsie and Ward taxonomic key (89), and their sex and gravid status were scored using a standard 10-40X dissecting scope. Fed and/or gravid (determined by the presence of an enlarged abdomen) females were excluded from analysis. A total of five host-seeking female mosquitoes were collected using an aspirator (BioQuip, Rancho Dominquez, California) at one time point at each site (Appendix J). Samples were immediately placed on ice. Individual mosquitoes were dissected to extract the tissues for genetic analysis. Before dissection, whole mosquitoes were surface sterilized with a 75% ethanol wash, followed by two rinses in sterile 1X Phosphate

Buffered Saline. The body of the mosquito placed on ice and dissected with EMS High Precision and Ultra Fine Tweezers (Electron Microscopy Sciences, Hatfield, PA) to expose the internal organs. Once identified, the crop, midgut, Malpighian tubules, and ovaries were dissected and removed. Each sample was pooled by tissue type at each site with 5 individuals per pool and site (i.e., 5 crops, midguts, Malpighian tubules, and ovaries per site) and immediately placed in a sterile 1.5 microcentrifuge tube in the -80^{o}C freezer for storage (n=79 samples). However, at Site 1, one pool of ovary sample was unable to be processed, and is why there are 79 samples.

Water, Soil and Plant Tissue Collection and Sampling

At each site, samples were collected from other areas of the environment which mosquitoes contact (plant, soil, and water). These samples provided sources of environmental bacteria from which DNA could be extracted. The environmental samples were collected within 2 meters of where mosquitoes were collected. Samples were collected in on the same day as the mosquitoes at each site in triplicate (i.e., 3 soil samples per site, 3 plant samples from each site, 3 water samples per site where possible). A total of 159 environmental samples were collected over the course of the study. Approximately 250 milligrams (mg) of soil were collected at the surface of the ground at each site with a clean metal spoon (i.e., surface sterilized with 70% ethanol prior to collection). Substrates that produce plant nectar, herein referred to as "Plant", were collected by rubbing flocked swabs (Puritan, Portland, TN) carefully pulling back the floral tissue (i.e., flower petals) and swabbing the area containing the nectaries. Plant juices from fruits (if present) were collected by carefully swabbing the liquid seeping from the fruit (if present) from each site (Appendix J). Flocked swabs were rubbed against the surface of either flower or fruit 10 times and rotated clockwise. Majority of plant samples was collected from flowers (n=55) as compared to fruit (n=5). Flowers and fruit were sampled from the same tree. One flower or fruit was collected per sample. Where possible, 250 µl of water samples were sampled in triplicate at the twenty sites with a sterile pipette from pooled water at each site (i.e., tree-holes, pooled water on the ground). All environmental samples were immediately placed on ice and stored in -80^{o}C freezer.

<u>*DNA extraction and sequencing*</u>

DNA was extracted from the samples of adult mosquito tissues, and bacteria within the water, soil, and plant nectar using a Qiagen MagAttract PowerSoil DNA Kingfisher Kit (Qiagen, Germany) on a KingFisher Flex (Thermo Fisher Scientific, Waltham, MA) following the manufacturer's protocol. Library preparation was done using a modified version of the Earth Microbiome Project 16S V4 Illumina Amplicon Protocol. Standard Earth Microbiome Project barcoded 16S primers were used, based off of 515F (Parada)-(5'-GTGYCAGCMGCCGCGGTAA-3') and 806R-(Apprill) (5'-GGACTACNVGGGTWTCTAAT-3') primers. Each PCR reaction contained the following components: 16.25 µL of nuclease-free water, 5.0 µL of 5X KAPA HiFi Fidelity Buffer, 1.0 µL of template DNA (approximately 10-87 ng), 0.75 µL of 10 mM KAPA dNTP Mix, 0.75 µL of 10 µM forward primer, 0.75 µL of 10 µM reverse primer, and 0.5 µL of 1 U/µL KAPA HiFi HotStart DNA Polymerase. A no-template PCR reaction was also performed as a negative reagent blank. PCR amplifications were performed in an Applied Biosystems SimpliAmp Thermal Cycler (Thermo Fisher Scientific, Waltham, MA) using the following conditions: initial denaturation at 95^{o}C for 3 min; 35 cycles of denaturation at 98^{o}C for 20 s, annealing at 60^{o}C for 15 s, extension at 72^{o}C for 30 s; and a final extension at 72^{o}C for 30 s. The PCR products were visualized on a 2% agarose gel before being purified and normalized to approximately 1.25 to 2.50 ng/□L using a Just-a-Plate kit (Charm Biotech, Cape Giradeau, MO). Purity and concentration of a subset of samples were assessed with a NanoDrop (Thermo Fisher Scientific, Waltham, MA). A volume of 10 µL from each sample was pooled and then purified using a 1.2X volume of homemade AMPureXP Beads (manufacturer, source). The library was checked for quality and quantity using a Bioanalyzer High Sensitivity chip (Agilent Technologies, Santa Clara, CA) and sequenced by Advanced Studies in Genomics, Proteomics and Bioinformatics at the University of Hawai‘i at Mānoa using Illumina MiSeq PE300 with a MiSeq Reagent Kit v3 (Illumina, San Diego, CA).

Bioinformatics

Bioinformatic processing of sequencing reads was performed using the bioinformatic pipeline to identify amplicon sequence variants (ASVs) (158). After quality control, sequencing resulted in 17.1 million reads (per sample mean: 59,749; per sample median: 60,128). Operational Taxonomic Units (OTUs) found in the reagent negative-controls and no-template PCR controls were removed from analysis as they likely represent contaminants along the molecular analysis pipeline. The OTUs were filtered per sample-type. The OTU taxa were removed if not seen in more than 1 time in at least 20% of samples using the “filter_taxa” function implemented in package Phyloseq in R (source or reference). The analysis generated OTU abundance per sample. Overall, 20 OTUs were from mosquito tissues, which was used for diversity measurements (species richness and Simpson’s index), nestedness temperature, modularity analysis, and to test for differences in the composition of microbiota within the mosquito host. The 919 OTUs for environmental samples were used to assess diversity measurements (species richness and Simpson’s index) and nestedness temperature. Detailed methods are described in the statistical analysis section.

Statistical Analysis

A generalized linear mixed model implemented in the package *glmmTMB* (133) in program R was used to test for differences in the composition of microbiota (OTUs) from host mosquitoes across tissue types (crop, midgut, Malpighian tubule, and ovary). A nestedness temperature (155), or a measure of the degree to which symbiont taxa found in species-poor samples are a nested subset of those found species-rich samples, was calculated to address whether environmental samples (soil, water and plant) or mosquito tissues (crop, midgut, Malpighian tubule, and ovary) serve as a microbial reservoir. Two weighted bipartite matrices were constructed: 1) environment + mosquito and 2) mosquito samples. Endosymbionts of *Wolbachia* (wAlbA and wAlbB) were excluded from the nestedness analysis as the focus of the analysis was to assess if environmentally acquired symbionts are a source for mosquito microbiomes (159). To account for disparities in OTU abundances between mosquito and environmental samples, the bipartite matrices were resampled without replacement using "rarefy_even_depth" function in phyloseq package in program R using "sample.size" 1556 for the environment + mosquito matrix and 2000 for the mosquito matrix. A bipartite matrix that had not been resampled was also used for nestedenss analysis as a comparison. Both bipartite matrices resulted in similar conclusions, and thus the data presented in Figure 4.3 was not resampled. The nestedness ranged from 0 (nested) to 100 (random) based on a weighted bipartite matrix, implemented in the "nestedtemp" function in vegan package in program R (134). Nested temperature was compared with a distribution of randomized null communities simulated using the "oesimu" function with the "quasiswap" method in vegan package. The Simpson's index of each mosquito and environmental microbiota, estimated with the function "diversity" in the package vegan. Richness was measured as the total number of OTUs in a sample.

I used the QuanBiMo algorithm, following the method and code described in Dormann et al. (157), to calculate modularity (Q) for the weighted mosquito tissue network. I then used the nullmodel function (100 randomizations; method 'vaznull') to convert Q to a z-score and estimate a p-value assuming one degree of freedom. The extent to which certain tissues select for microbes was calculated using the network wide H2 index under the "H2fun" in package vegan (160). The H2 index returns a value from 0 (generalized) to 1 (specialized). Deviations from null expectations were quantified using a distribution of null community matrices calculated using the quantitative r2table method in package vegan (161).

RESULTS

<u>*Mosquito and Environmental Microbiome*</u>

A total of 16,495 bacterial OTUs (15,855 from the environmental samples; 640 from the mosquito tissues) were identified from the pooled tissues of 79 *Ae. albopictus* mosquito crop (n=20), midgut (n=20), Malpighian tubules (n=20), and ovaries (n=19) and 159 environmental samples (soil, plant, and water). A total of 919 bacterial OTUs had a prevalence greater than 20% in each sample type (environmental or mosquito). This included 21 phyla: Acidobacteria, Actinobacteria, Bacteroidetes, Chloroflexi, Cyanobacteria, Deinococcus-Thermus, Elusimicrobia, Entotheonellaeota, Firmicutes, Fusobacteria, Gemmatimonadetes, Latescibacteria, Nitrospirae, Planctomycetes, Proteobacteria, Rokubacteria, Spirochaetes, Tenericutes, Thaumarchaeota, and Verrucomicrobia. Of the resampled bacterial OTUs, 185 families were identified (Figure 4.1). Of the resampled OTUs, the number of predominant OTUs by sample type are as follows: 822 soil OTUs; 68 plant OTUs; 42 water OTUs; and 20 mosquito tissue OTUs. OTU co-occurrence was noted between environmental and mosquito sample types (Table 4.1 and 4.2, Appendix K).

Table 4.1: OTU co-occurrence of environmentally acquired symbionts amongst mosquito tissue types. The number in parentheses below each tissue is the total number of OTUs in each tissue. Numbers in parenthesis indicate the proportion of the tissue specific microbiome that is shared with the environmental microbiome. Vertically transmitted symbionts wAlbA and wAlbB were removed to curate this table. Data in the table corresponds to full datasets that have not been resampled.

	Crop (N=12)	**Midgut (N=11)**	**Malpighian Tubule (N=14)**	**Ovary (N=12)**
Plant	4 (0.3)	3 (0.27)	4 (0.29)	5 (0.41)
Water	2 (0.16)	1 (0.09)	2 (0.14)	2 (0.17)
Soil	2 (25)	2 (0.18)	3 (0.21)	3 (0.25)

Table 4.2: The number of OTUs that co-occurrence amongst environmental sample types (Plant, Water, and Soil)

	Plant	**Water**
Plant		
Water	6	
Soil	14	11

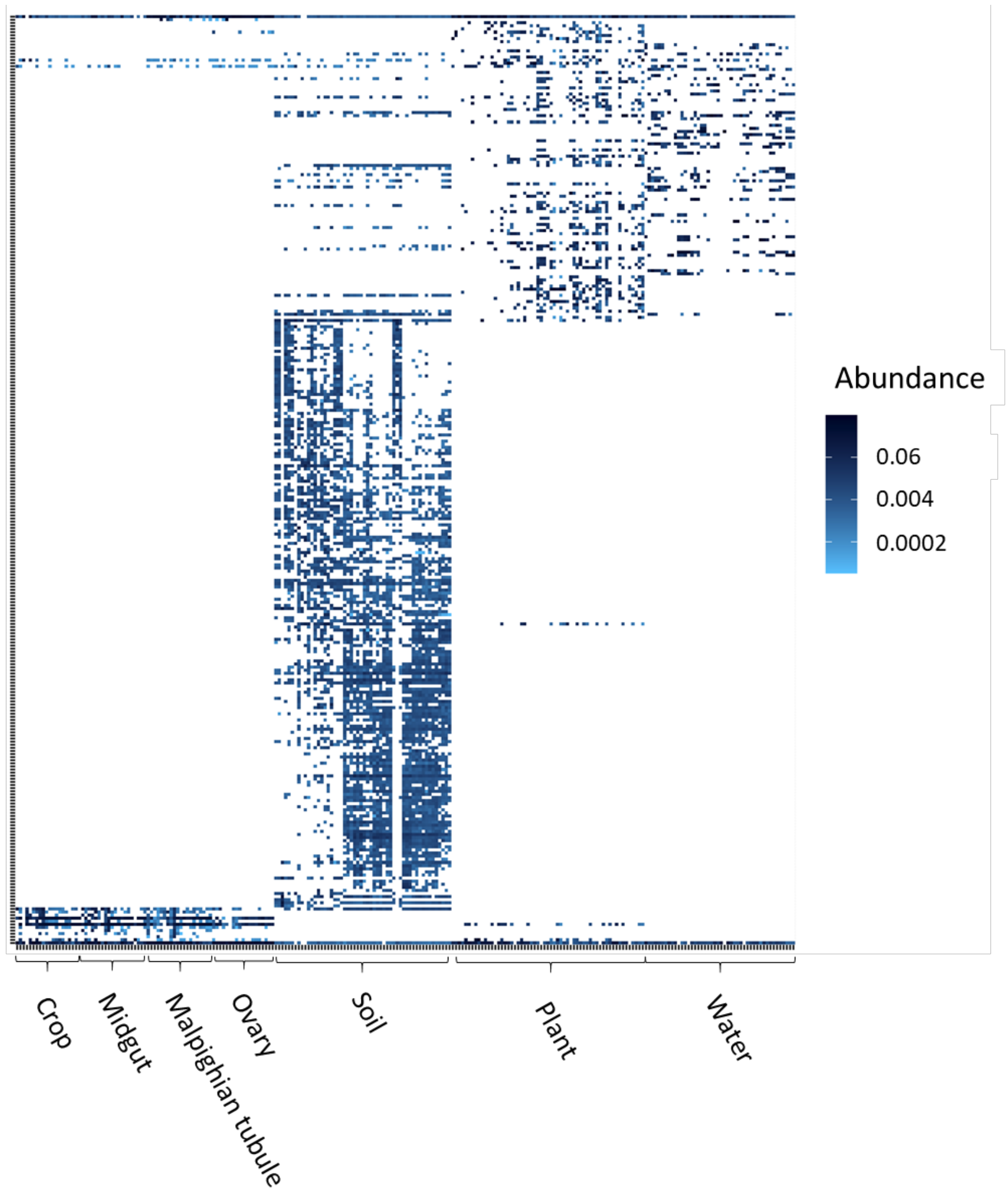

Figure 4.1: Heatmap showing the prevalence of bacterial taxa within environmental samples (Soil, Plant, and Water) and mosquito tissues (Crop, Midgut, Malpighian Tubules, and Ovaries). Cell values are calculated as proportions across rows.

Mosquito Microbiome

Microbiome composition varied significantly across tissue types ($p < 0.0001$, Figure 4.2, Appendix L, M, N). The conditional modes of bacterial symbionts in mosquito tissues indicate varied responses to tissue environment (Appendix N). For instance, the conditional modes of symbionts found in the crop indicate that the symbionts *Acidibacter* (midas_o_26:midas_f_98), ERWIN1, CAULO1, *Mesorhizobium* (Hyphomicrobiales:Phyllobacteriaceae) , CHITI1, *Bradyrhizobium*, *Sphingomonas*, *Burkholderia*, *Asaia*, and ACIDO1 are more abundant, relative to the population average across all tissue types (i.e. crop, midgut, Malpighian tubule, and ovaries). Interestingly, ACIDO1 was entirely restricted to the crop (Appendix L). While the midgut shared some similar bacterial taxa to the crop, ERWIN1 lineage was entirely absent from the midgut tissue, and subsequently had a negative conditional mode (-3.0). While the Malpighian tubules and ovaries shared some bacterial taxa (wAlbA, 89wAlbB, HALO1, and *Ricketsiella*), *Spinghomonas* and *Vibriomonas* were absent from the ovaries. The ovaries were also the only tissue types to harbor symbionts *Acinetobacter*, PLAN1, and *Cloacibacterium* (Appendix L, M and N).

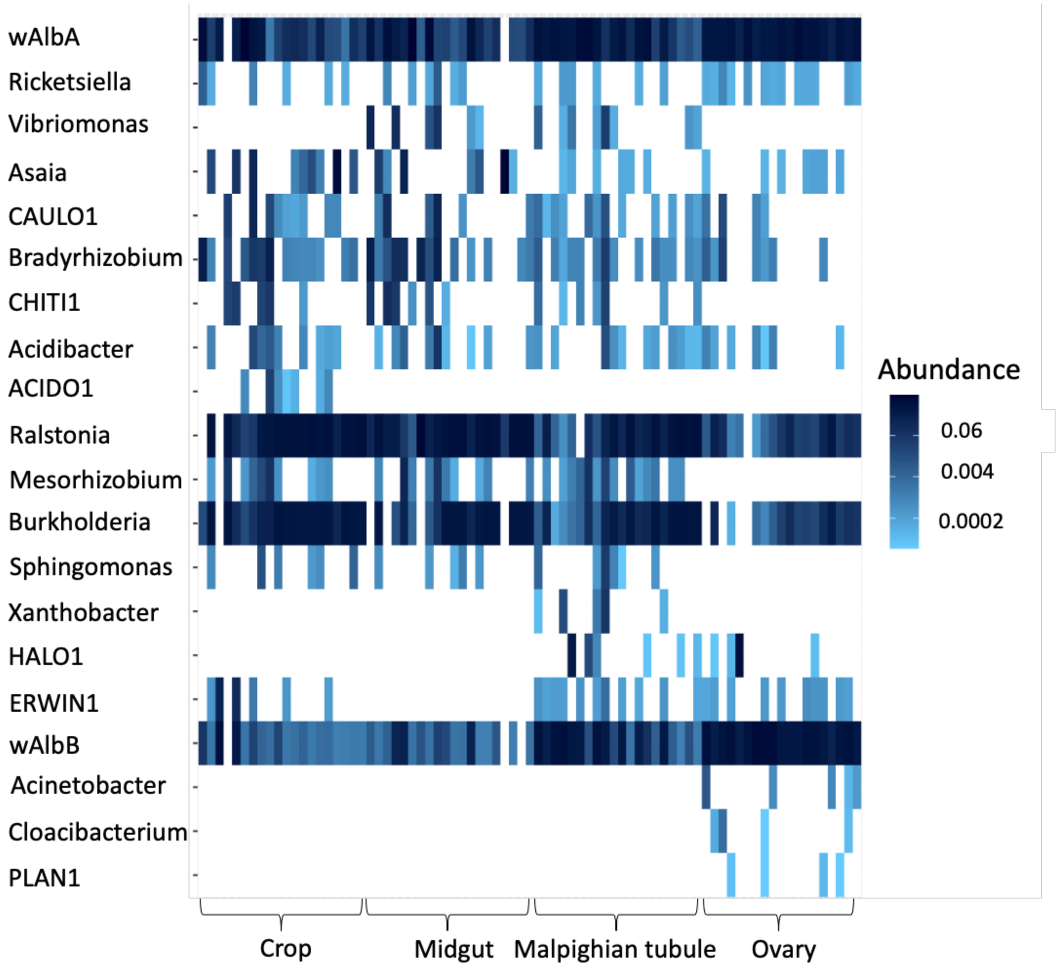

Figure 4.2. Heatmap showing the prevalence of bacterial taxa distributed across *Ae. albopictus* tissues (crop, midgut, Malpighian tubules, and ovaries). Cell values are calculated as proportions across rows. Some of the OTU names had multiple hits when blasted against the NCBI GenBank database and have been given acronyms for clarity. CAULO1 lineage were most similar to Caulobacteraceae, CHITI1 lineage were most similar to Chitinophagaceae, ACIDO1 lineage were most similar to Acidobacteriia, HALO1 lineage were most similar to Halomonadaceae, ERWIN1 lineage is most similar to Erwiniaceae, and PLAN1 lineage was most similar to Planctomycetes.

Mosquito microbiome is nested within the environment.

The resampled environmental OTUs which exclude vertically transmitted symbionts had an average (± standard error of the mean) species richness of 5.94 ± 0.014 and evenness (Simpson's index) of 0.99 ± 0.002. The resampled mosquito OTUs had an average (± standard error of the mean) species richness of 1.6 ± 0.004 and evenness (Simpson's index) of 0.77 ± 0.002. Ecological network analysis indicated that *Ae. albopictus* tissue microbiomes are nested within environmental samples as compared to null expectations (Nested Temperature= 19.7, p= 0.001) (Figure 4.3)

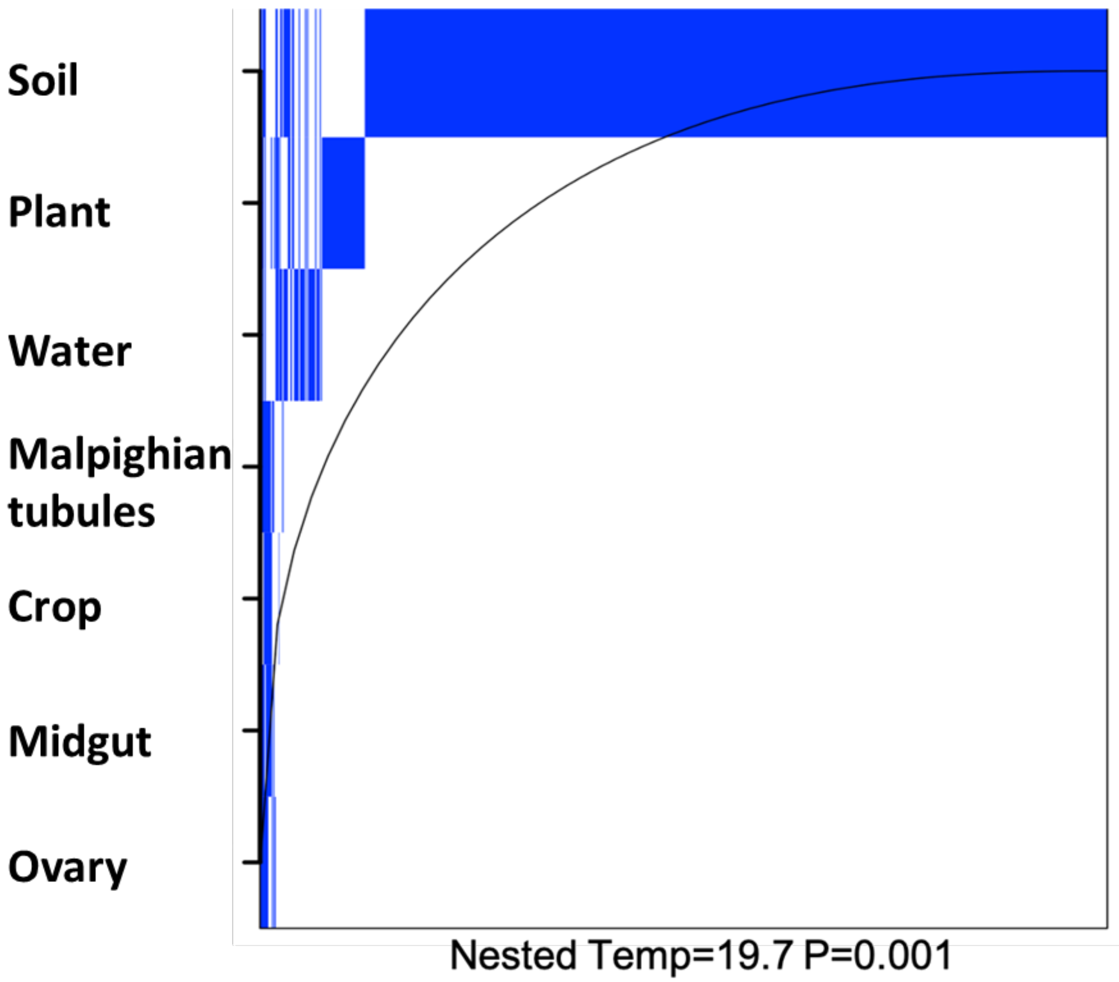

Figure 4.3: Nestedness plot of bacterial microbiome aggregated by environmental sample and mosquito tissue types. Bacterial taxa presence in each sample type is represented as a blue rectangle. Bacterial taxa are organized by residence (left to right) across the different sample types, and rows are ordered by the highest species richness to the lowest species richness (top to bottom). The curved line represents the "Fill line". If all bacterial taxa occurred above the "Fill line", then the system would be considered to be perfectly nested.

Ecological network analysis also suggested that mosquito tissue microbiomes are not nested across tissues consistent with null expectations (Nested Temperature= 25.4, p= 0.5) (Figure 4.4), despite tissue OTU-co-occurrence interactions along the alimentary canal (with exception of ovaries). While mosquito tissues derive part of their microbiome from environmental sources, specialization network analysis (H2'=0.16, p =0.001) indicated that mosquito tissues are specialized as compared to null expectations. Network modularity assesses link rich clusters in a community and allows identification of species assemblages across ecological communities (186). My analysis indicated that mosquito tissues were modular (Q=0.24, z value=315, p= 0.0001) as compared to null expectations (Figure 4.5). One module was shared between the crop and midgut tissues, and included several symbionts such as *Ralstonia*, *Burkholderia, Asaia*, *Bradyrhizobium*, CAULO1, CHITI1, *Mesorhizobium*, *Spingomonas*, ACIDO1, *Acidibacter*, and *Vibriomonas* (Figure 4.5). A second module was shared between the Malpighian tubule and ovary tissues, and included the symbionts wAlbA, wAlbB, ERWIN1, Ricketsiella, HALO1, Xanthobacter, Acinetobacter, PLAN1, and *Cloacibacterium* (Figure 4.5).

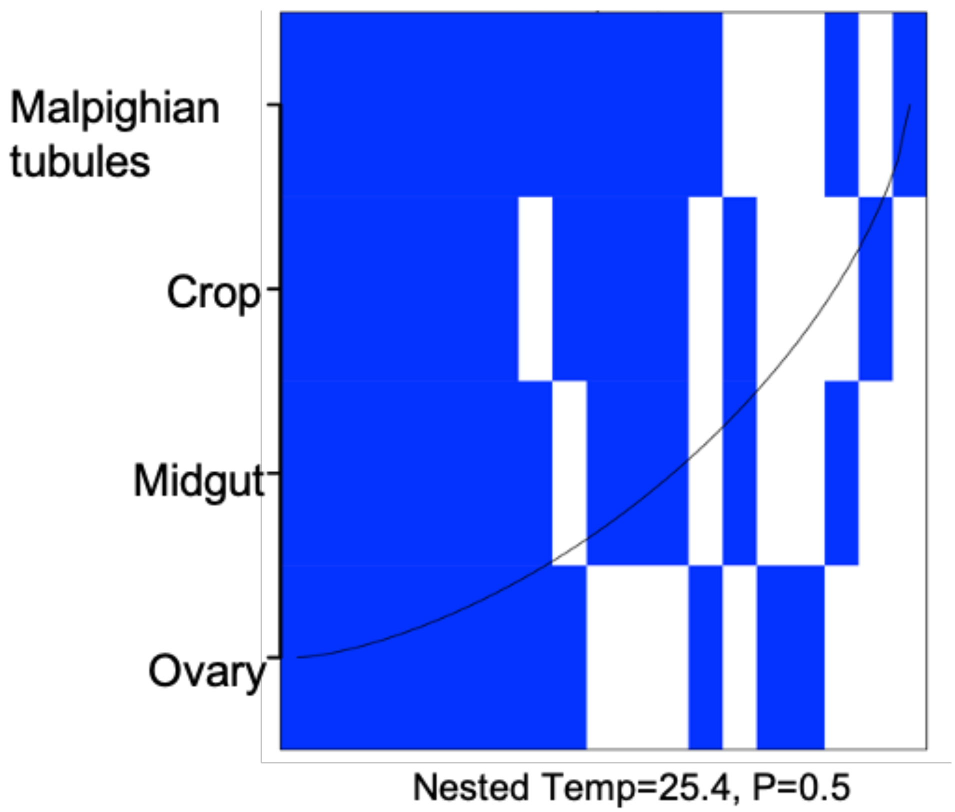

Figure 4.4: Nestedness plot of bacterial microbiome aggregated by mosquito tissue types. Bacterial taxa presence in each sample type is represented as a blue rectangle. Bacterial taxa are organized by residence (left to right) across the different sample types, and rows are ordered by the highest species richness to the lowest species richness (top to bottom). The curved line represents the "Fill line". If all bacterial taxa occurred above the "Fill line", then the system would be considered to be perfectly nested.

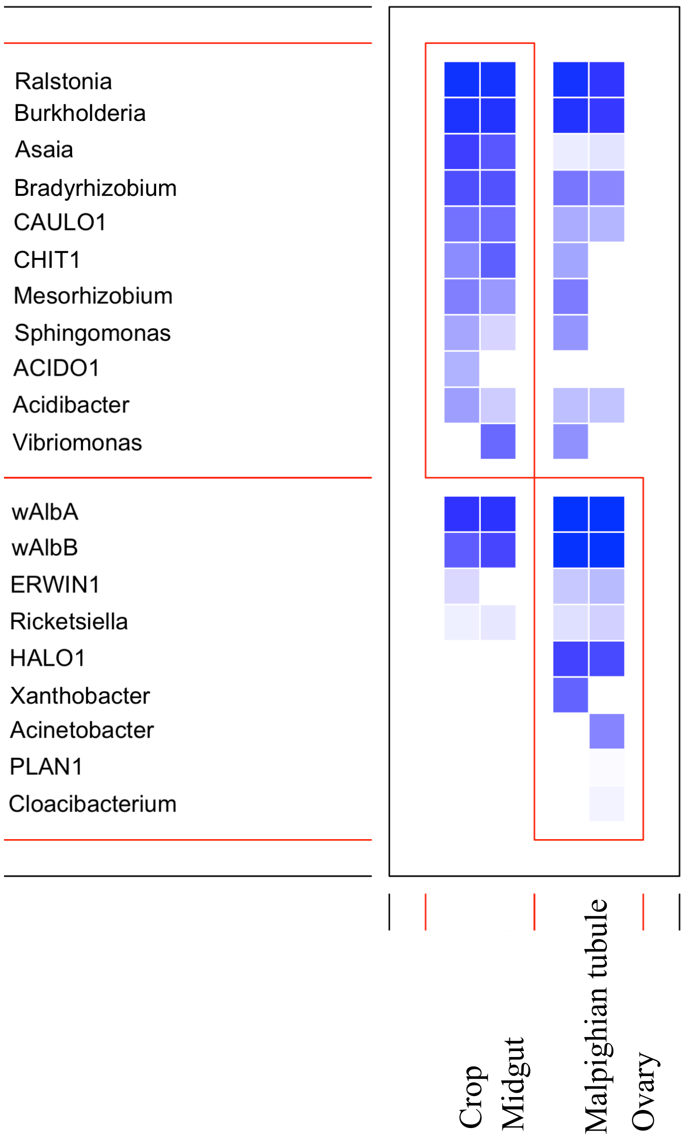

Figure 4.5. Interaction matrix of co-occurring bacterial symbionts within mosquito tissues (crop, midgut, Malpighians tubules, and ovaries) and its respective modules. Darker squares indicate more frequent co-occurrences. Red boxes indicate the two detected modules present amongst mosquito tissues. Ralstonia, Burkholderia, Mesorhizobium co-occur readily within crop and midgut, while wAlbA, wAlbB, co-occur frequently within the Malpighian tubule and ovary.

DISCUSSION

Mosquito microbiome composition is dynamic, with substantial diversity and variation both within and among wild mosquito populations. Given the influence the microbiome has on vectorial capacity, understanding factors that contribute to microbiome variation are essential to developing strategies that aim to reduce mosquito-borne disease transmission. While several studies have attributed microbiome variation to individual-physiological states (i.e., species, blood-fed status, sex, host immunity, co-symbiosis) and habitat (i.e., geographic habitat) (129, 162), the very origin of mosquito microbial symbionts from the environment and how they assemble within hosts to form a microbiota is inadequately understood. Here, I clarify the origin of mosquito microbial symbionts by *i*) investigating environmental sources of potential microbial symbionts that mosquito host interact with, and *ii*) explore how the community of the mosquito microbiome varies within the host (i.e., distinct tissues). Broadly, these data demonstrate that mosquito tissue microbiomes are nested within environmental sources (soil, plant, and water), indicating that mosquito microbiotas may be sourced from specific environmental sources like plant, soil, and aquatic habitat (i.e. water)(163). Additionally, while mosquito microbial symbionts derive from environmental sources, they also assemble into distinct microbiomes across tissue types (crop, midgut, Malpighian tubule, and ovaries) and form specialized modules based on commonly co-occurring symbiont species across tissue groups (i.e., digestive tissues of the crop and midgut and the Wolbachia-harboring Malpighian tubules and ovaries). Several recent studies have demonstrated environmental influence on the microbiome composition of mosquitoes. For instance, *Cx. quinquefasciatus* bacterial symbionts were associated with aquatic habitats, demonstrated by shared bacterial symbionts found in both sources (64). Previous studies have also described nestedness in other microbial systems, in which a system with reduced alpha-diversity subsets from a system with greater alpha-diversity (161, 164). The overall pattern demonstrated by these data is that low diversity microbiomes (i.e., mosquito microbiotas) are nested (i.e., subsets) within high diversity microbiomes (i.e., water, soil, plants). The data suggest that environmental samples (water, soil, plants) are a source for mosquito symbionts, and the differences in diversity may be associated with environmental filtering and other processes like dispersal ability. Indeed, only a small proportion of symbionts from the prospective cumulative diversity co-occurred within environmental and mosquito samples, which indicates that only certain symbionts are capable of colonizing the mosquito host, which is likely due to diverse ecological processes driven by host and microbe-microbe interactions within the assembling mosquito microbiomes. Alternatively, these results cannot reject the hypothesis that microbes are colonizing mosquitoes from unsampled reservoirs that share similar symbionts (i.e., air, skin of vertebrate animals).

Several bacterial symbionts found within mosquitoes were also detected in environmental samples. For example, the bacterial symbionts *Ralstonia*, ERWIN1, *Asaia*, *Ricketsiella*, and *Acinetobacter*, were detected in plant samples; ERWIN1, and *Ricketsiella* were detected in water samples; and ERWIN1, *Ricketsiella*, and *Bradyrhizobium* were found within soil samples. These results suggest that mosquito symbiont acquisition may occur via interactions with environmental sources through feeding in its immature stages in aquatic habitats, or in its adult phase via foraging, host-seeking, resting, and reproductive behavior. While not assessed in this dissertation, previous studies have also highlighted that mosquito microbiomes are influenced by hematophagous behavior, which results in a dramatic reduction in bacteriome diversity (162). Additionally, the bacterial symbiont *Asaia*, which is present in *Anopheles stephensi,* is likely acquired from the nectar in tropical flowers, and associated with foraging behavior(165).

While the overall mosquito microbiome was nested in the environmental microbiome, each mosquito tissue was comprised of a distinct composite of bacterial symbionts, highlighting that the community of the mosquito microbiome occurs differentially across distinct tissues with specific functions to the host organism. My data demonstrates that microbes were significantly specialized within mosquito tissues and organized into two modules of highly co-occurring bacterial symbionts: those in the digestive tissues of the crop and midgut and those in the Wolbachia-containing tissues of the Malpighian tubules and the ovaries.

Multiple hypotheses might explain the apparent tissue specialization and modularity identified in this study. There is growing evidence that hosts actively maintain symbiosis that will support host physiological processes (166, 167). Indeed, it is well known that symbionts provide essential functions to their host (168). Selection may favor hosts that differentially associate with symbionts and harbor them in the right tissues to provide these functions. Some of my observations indicate that this may drive the formation of modules observed in this study. For instance, my data demonstrates a module between the microbiomes of the crop and midgut tissues, two tissues that have important functions associated with food digestion and nutrition (169, 170). The symbiont *Burkholderia* found within the crop and midgut has been shown to assist with nutritional functions for stinkbugs, and can even confer resistance to insecticides (171). While the role of *Burkholderia* in mosquito digestion remains unresolved, this symbiont has been found in other mosquito midguts, as well as symbionts *Ralstonia* and *Asaia* (111). Indeed, *Asaia*, is commonly found in nectivorous arthropods such as mosquitoes (67), and may impact male mosquito fitness (61, 67) and the development of *Plasmodium spp*. parasites in mosquito host (67). Given the shared physiological processes of the crop and midgut (i.e., nutritional functions) and close proximity to each other (i.e., connection via alimentary canal) it is likely that the host would select for symbionts capable of supporting this broad physiological function and that connectivity via the alimentary canal allows for the close interactions of symbionts between these tissue types. For instance *Bradyrhizobium* has been shown to have nitrogen fixing properties, which may be used to detoxify ammonia after a blood meal (109).

Module formation may recapitulate developmental processes. For example, Malpighian tubules are excretory tissues in mosquitoes responsible for osmotic balance, and excretion of waste, they are also known to remain intact during metamorphosis in *Drosophila* (139, 172). Recent studies have highlighted the possibility that Malpighian tubules facilitate the transstadial transmission of bacterial symbionts from the larval to the adult stage (139). Indeed, in this study the Malpighian tubules have the greatest species diversity as compared to the crop , midgut , and ovaries (Table 4.1), and may serve as a source pool for transstadial transmission of environmentally acquired bacterial symbionts to redeveloped tissues following metamorphosis. The Malpighian tubules served as refugium for the bacterial symbiont *Pseudomonas*, facilitating its transmission from larvae to adult in *Anopheles stephensi* mosquitoes (139). The Malpighian tubules also serve as a site for the pathosymbiont *Ascogregarina taiwanensis*, which is transtadially transmitted (120). Previous studies have also demonstrated that Malpighian tubules harbor large numbers of Wolbachia bacteria and may persist through vertical transmission (172). It is possible that the derivative tissues of the Malpighian tubules may serve as a source pool for vertically transmitted bacteria like *Wolbachia*, or may play an important role in host fitness.

While a host environment plays an important role in microbiome construction and maintenance, microbial growth optima determine the magnitude of microbial growth and distribution amongst mosquito tissues. For instance, ACIDO1, which is known to contain acidophilic strains (169), was indeed entirely restricted to the crop, which is an acidic environment due to high concentrations of carbohydrates. Furthermore, the ovaries where the only site for bacterial symbionts *Acinetobacter*, PLAN1, and *Cloacibacterium.* This was surprising in that *Cloacibacterium*, has been shown in the midgut of *Ae. albopictus* mosquitoes (16). Overall, these factors determine not only microbial survival but colonization potential dependent on tissue structure and the physiological environment as well as neighboring microbes and the intra-microbial dynamics of mosquito microbiotas.

This study has identified that *Ae. albopictus* mosquitoes derive a part (30%) of their microbiome from environmental sources and these microbes assemble differentially among distinct tissues. Clarifying the origin of diversity, and how various anatomical compartments of medically relevant mosquitos influence microbiome composition will inform my understanding of mosquito physiology, development, immune function, and cumulatively, vectorial capacity. Furthermore, understanding which tissues support certain symbiosis will likely inform which symbionts are potential targets for paratransgenic studies and suggest how these paratransgenic platforms might disperse between hosts in real landscapes.

CHAPTER 5

GENERAL DISCUSSION

Despite current efforts to manage medically important mosquitoes, mosquito-borne diseases are emerging worldwide and are an ever-present threat to human and zoonotic health (47, 173). Furthermore, mosquitoes populations and species distributions are expected to change as global temperatures rise (141). This may increase mosquito-borne disease transmission. Unique in their high endemism, biodiversity and year-round suitable climate, the islands of Hawaiʻi support non-native mosquito populations (31, 174) which have negatively impacted native birds and humans (37, 102). As in other parts of the world, surveillance programs in the state of Hawaiʻi are underfunded and management programs rely on limited technologies.

An alternative strategy toward reducing mosquito-borne disease transmission relies on understanding how the environment influences mosquito distribution and its host-associated microbiome. Previous studies have highlighted the impact the microbiome can have on the vectorial capacity in mosquitoes (175). For instance, one approach uses the bacterial endosymbiont *Wolbachia* to reduce mosquito populations or create mosquitoes that are incapable of transmitting Dengue virus (176). Bacterial symbionts are also a primary target for disease reduction platforms like paratransgenesis (145). Paratransgenic platforms center around the modification of symbiotic microbes that will deliver anti-pathogen molecules to decrease mosquito-borne disease transmission within the mosquito host.

While these disease reduction strategies offer a novel approach to reducing mosquito-borne disease transmission, significant gaps in knowledge about the factors that influence mosquito populations and their host associated microbiomes exist. These gaps limit the development and likely success of these techniques in the field. For instance, the biotic and abiotic factors that influence mosquito populations are poorly understood, particularly on islands. Studies that investigate seasonality and environmental gradients are critical to understanding factors that influence mosquito populations. Additionally, a deep understanding of regional mosquito microbiomes is needed to *i*) identify how mosquito microbiomes vary across time and space to further understand how abiotic factors influence symbiosis., *ii)* identify biotic factors that influence mosquito microbiome composition, such as co-symbiosis, *iii*) further understand microbiome assembly within the mosquito host, specifically how symbionts disseminate and establish within the distinct tissues of mosquitoes, *iv*) identify components of the environment that act as a source for mosquito symbionts.

Aedes albopictus is a globally distributed vector of arboviruses and is widespread throughout Hawaiʻi. This dissertation addressed three objectives to further understand drivers of variation in *Aedes albopictus* and its microbiome in Hawaiʻi.

OBJECTIVE 1: DESCRIBE THE DISTRIBUTION OF ESTABLISHED MOSQUITOES ON THE ISLAND OF MAUI IN HAWAI'I OVER THE COURSE OF A YEAR (CHAPTER 2).

Currently, there are six species of blood-feeding mosquitoes that are believed to be established in the state of Hawaiʻi: *Aedes albopictus*, *Aedes aegypti*, *Aedes vexans*, *Aedes japonicus*, *Culex quinquefasciatus*, and *Wyeomyia mitchellii*. Despite their capacity to transmit mosquito-borne diseases, their exact distribution and factors influencing their populations are poorly resolved (11). In this dissertation, the patterns of mosquito distribution were evaluated in relation to climate variables (rainfall and temperature), elevation, space, and time (Chapter 2). Understanding what mosquitoes are present in a given area and their distribution is key in determining both the risk of disease outbreak and in implementing disease mitigation strategies.

In a yearlong survey, four species of mosquitoes on the island of Maui were identified: *Aedes albopictus*, *Aedes vexans*, *Culex quinquefasciatus* and *Wyeomyia mitchellii* (Chapter 2). *Aedes albopictus* was found to be widely distributed and abundant over much of the island and present year-round, whereas the other mosquitoes were not. The habitats sampled across this study differed in their proximity to human habitation, available vegetation, and climate type (Chapter 2, Table 2.1), These differences likely contributed to the variation in abundance and distribution of the different species collected in this study.

Aedes albopictus abundance (measured by adult capture) and oviposition (detection of presence or absence of eggs) varied geographically and over time, but the pattern of variation was not consistent between the two measures (adults and eggs). *Aedes albopictus* oviposition shifts in accordance with climate variables (temperature and rainfall) and elevation (Chapter 2). Oviposition was driven by warm low elevation sites (Chapter 2). Surprisingly, the abundance of *Ae. albopictus* adults was uncorrelated with climate variables (rainfall and temperature) and elevation though there was spatiotemporal variation. Where *Ae. albopictus* was present across most sites around Maui, there does not seem to be a limitation on the types of environments this species will occupy and is right now limited only in terms of elevation. For instance, *Ae. albopictus* was not found at sites Haleakala National Park, Haleakala Road 1 and 2, which are high elevation (range 1131-2051 meters; Chapter 2, Table 2.1).

Culex quinquefasciatus was found to be abundant but had limited distribution as compared to *Ae. albopictus*. There were limited collections of *Ae. vexans* and *W. mitchellii*. Detections of these species may be seasonal, based on habitat, and a mismatch between sampling protocols and mosquito ecology.

Overall, these findings demonstrate that not all mosquito species are evenly distributed across the island of Maui. Even the most widespread species are limited below an elevational threshold. The findings suggest there may be potential expansion of mosquito distribution due to increasing global temperatures. Higher temperatures could enable movement of mosquitoes to upper elevations. It is predicted that as the climate changes, there will be changes to the environment may have a substantial impact to species distributions (7, 177). Native species of other organisms may be displaced and or negatively impacted by invasive species like mosquitoes, particularly if they are better adapted to the changing climate. Climate changes may increase the ability of mosquitoes to expand their range (i.e., expanding into high elevations) leading to greater susceptibility of avian malaria to native birds who live in higher elevations (36). Several factors were not assessed in this study that may influence mosquito distribution: land use, proximity to human habitation, and available vegetation. These factors may impact the distribution of mosquitoes.

These findings enhance the capability of implementing vector control strategies by *i*) providing coordinates of where medically important mosquitoes occur, creating the ability to rapidly respond to a mosquito-borne disease outbreak, and *ii*) provides a baseline to understand how mosquito distributions change with time and to determine if new incursions occur. While these findings are beneficial to vector control programmes, the impact of factors like the microbiome, which either enhance or inhibit mosquito population and reproduction need to be understood. More detailed, consistent and long-term sampling will create a more comprehensive data set from which to explore these patterns.

OBJECTIVE 2: EVALUATE THE VARIATION IN THE MICROBIOME OF ADULT AEDES ALBOPICTUS (A) ACROSS THE ISLAND OF MAUI AND (B) BETWEEN MOSQUITOES AT EACH SAMPLING LOCATION (POPULATION) OVER TIME. (CHAPTER 3).

There is substantial variation in microbiome composition within and among mosquito populations, however the drivers of this variation are not well understood. Given impact the microbiome has on influencing vectorial capacity in mosquitoes, clarifying factors that contribute to microbiome variation are crucial to future disease prevention strategies. The specific environmental and ecological factors that influence diversity in the bacteriome of *Aedes albopictus* on the island of Maui were investigated (Chapter 3). The microbiome of *Aedes albopictus* on Maui varies greatly across geographic location but was uncorrelated with climate variable (temperature and rain) and elevation (Chapter 3). This finding suggests that environments have a distinct source pool of symbionts that contribute to variation in the microbiome of mosquitoes across sites. The variation across geographic location and lack of correlation to climate variables was surprising. As with the assessment of mosquito distribution (Chapter 2), several factors (i.e., land use, proximity to human habitation, available vegetation) that may contribute to this variation were not specifically measured and may be a factor driving variation in the mosquito microbiome.

The composition of the *Ae. albopictus* microbiome is correlated with the host's intensity of infection with the pathosymbiont, *Ascogregarina taiwanensis* (Chapter 3). Thus, co-occurring microorganisms may impact the diversity and variation of the microbiome in a disease-transmitting mosquito. Given that the microbiome of mosquitoes is being investigated for developing disease mitigating strategies, this finding suggests that these platforms should investigate how co-occurring microbes will impact these strategies. For instance, paratransgenesis uses certain microbes that will prevent mosquitoes from transmitting mosquito-borne pathogens. The ideal microbe should have the ability to persist within mosquito host in diverse environments and resist impacts from co-occurring microorganisms, diverse habitats or distinct tissue environments found within the mosquito host.

OBJECTIVE 3: CLARIFY THE ENVIRONMENTAL SOURCES THAT SERVE AS SOURCES OF MOSQUITO SYMBIONTS IN ADULT AEDES ALBOPICTUS AND CHARACTERIZE HOW ENVIRONMENTALLY ACQUIRED SYMBIONTS PARTITION AMONG DISTINCT TISSUES (CHAPTER 4).

A key feature to mosquito microbiotas is the substantial ecological diversity among individual host microbiomes. Geographical location and infection intensity of a co-occurring microorganism such as *Ascogregarina taiwanensis* has been shown to influence the diversity and variation in mosquito microbiomes (Chapter 3). While these finding are important, the environmental sources of these symbionts that mosquitoes interact with and how they assemble within the mosquito host still remains unclear. The distinct mosquito tissues offer diverse environments for potentially colonizing symbionts.

When evaluating the relationships between the environment a mosquito develops in and the microbiome within the different organs it was found that the tissue microbiomes are nested (i.e., subset) within environmental (soil, plant, and water) sources that they interact with through foraging, resting and reproductive behavior (Chapter 4, Figure 4.3). This finding indicates the microbiome of mosquitoes is indeed partially sourced from the environment, and further corroborates the findings that mosquito microbiomes are affiliated with geographic site (Chapter 3). Additionally, while a portion of the *Ae. albopictus* microbiome derives from the environment (Chapter 3), they sort differentially amongst the distinct tissues (crop, midgut, Malpighian tubule, and ovary) and form two specialized modules (crop/midgut and Malpighian tubules/ovaries) (Chapter 4). Presumably, these specialized modules form based on microbe niche preferences and/or selection of microbes that will provide support for a physiological process but as yet the specific mechanisms of this assembly process remain poorly resolved, and could be related to *i*) microbe-microbe interactions, *ii*) niche effects, and *iii*) and host-mediated microbe selection.

Additionally, it may be those organs such as the Malpighian tubules could serve as a source pool for vertically transmitted symbionts like *Wolbachia*. Furthermore, the variation in microbiome composition across *Ae. albopictus* host tissues may be a direct result of differential different growth requirements that symbionts have and likely plays a significant role in the establishment of symbiosis within the distinct mosquito tissues. Broadly, the data suggest that the diversity and variation of *Ae. albopictus* microbiome sources from the environment in which mosquitoes interact with, and that tissue specialization is likely a result of complex processes that involve host and microbe-mediated effects.

CONCLUSION

The globally distributed vector, *Aedes albopictus* was found to be widespread through the island of Maui and varied seasonally and across the distinct habitats. Climate variables (rainfall and temperature) and elevation influence the oviposition of *Ae. albopictus*, but not adult abundance. *Aedes albopictus* may have the potential to increase its population in sites that are warm and low elevation and may result in some areas being more at risk for mosquito-borne disease transmission. While climate variables and elevation explained certain aspects of *Ae. albopictus* population dynamics (i.e., oviposition), they likely did not fully capture the complexity of the habitat in any one space or time. Other factors like the microbiome, may influence mosquito populations.

The microbiome of *Ae. albopictus* varied across geographic location and infection intensity with a co-occurring symbiont *Ascogregarina taiwanensis*. A deeper look into environmental sources highlighted that a portion of mosquito microbiotas derive from the environment, and partition differentially across mosquito tissues (crop, midgut, Malpighian tubules, and ovaries). Taken together, the resulting data implies that a holistic approach to understanding mosquito vectors should be taken. Further investigating how climate, habitat, and seasonality influence mosquito vectors and their microbiome can benefit mosquito control strategies.

FUTURE RESEARCH

Future studies that will elucidate factors that influence mosquito vectors, and their microbiome should incorporate various geographic locations. While the island-wide survey gave insight into the influence of geographic site and seasonality on *Aedes albopictus* population dynamics (Chapter 2), a more intense sampling regime and incorporation of other understudied islands may yield different patterns. Furthermore, a deeper look into other factors such as proximity to human habitation, available vegetation, and incorporation of other climate variables would be beneficial, and would provide insight into how ecological factors influence mosquito population dynamics. Do factors such as wind speed, solar radiation, vegetation type, and canopy wetness influence mosquito populations? Further understanding the relationship between these factors and mosquito diversity, distribution and abundance can shed light on how mosquito populations may be influenced by climate change.

Various factors of the environment that were not measured in this dissertation also likely impact the diversity and variation in the mosquito and its microbiome. Natural factors of the environment (soil, water, plants) serve as a microbial species pool for *Ae. albopictus* mosquitoes

(Chapter 4). However, other factors within the environment may also impact mosquito microbiomes. In the wild, mosquito eggs are oviposited into aquatic environments that vary based on organic material (i.e., amount of detritus). Organic material in aquatic environments is largely composed of plant material which contributes to the microbiome diversity, nutrient availability, pH, and metabolite composition. Secondary metabolites from plants may alter life history traits of mosquitoes (i.e., fecundity, pupation rate, size, longevity, and abundance) and may impact the diversity and variation of mosquito microbiomes by selection for certain microbes. Do plant secondary metabolites influence the diversity and variation of mosquito microbiomes? Or are the secondary metabolites acting indirectly, and altering the environment (i.e., pH) of aquatic habitats, and either limiting or enhancing the abundance of certain microorganisms? Additionally, if the landscape if dominated by either native or invasive plant species, will this influence the life-history trait of mosquitoes and their microbiome? For instance, a recent study has shown that soil microbiome can facilitate the establishment of invasive plants (178). Do environments dominated by invasive species enhance mosquito populations (i.e., increase reproduction and fecundity), and impact their microbiome? Additionally, many environments contain artificial containers (i.e., plastic containers, tires) depending on their proximity to human habitation. A study has shown that *Ae. albopictus* exhibited high tolerance tire leachate (i.e., barium, cadmium, zinc), and may facilitate the reproduction of *Ae. albopictus* (179). Future studies should investigate the role artificial containers have on life-history traits of mosquitoes and their host associated microbiome.

Interdomain interactions contribute to the diversity and variation of mosquito microbiomes (Chapter 3). However, the exact mechanisms of this remain unknown. Does the presence of certain symbionts promote or inhibit the establishment of potentially colonizing symbionts? Full factorial experiments that manipulate infections with diverse symbionts would characterize the interactions elicited by co-occurring symbionts and resolve the processes that contribute to diversity in the microbiome of mosquitoes and enable study of the assembly process.

This dissertation and other studies have highlighted the role of the environment in influencing the distribution of mosquitoes and their microbiome. However other studies have demonstrated variation in the microbiome by mosquito species, among individuals of conspecifics, and wild versus laboratory reared (64). Several processes may be responsible for this, and could be niche, host, microbe or stochastically driven. Given the impact the microbiome has on the vectorial capacity of mosquitoes, and its potential role in creating disease mitigating platforms, further understanding factors that drive mosquito abundance, distribution, and microbiome composition are extremely important in lowering the global disease burden caused by mosquitoes.

APPENDICES

APPENDIX A: AVERAGE TEMPERATURES, AND RAINFALL FROM 18 SITES ACROSS MAUI, HAWAI'I

Site	April Temp. (°C)	April Rain. (mm)	June Temp (°C)	June Rain. (mm)	August Temp. (°C)	August Rain. (mm)	October Temp. (°C)	October Rain. (mm)	December Temp. (°C)	December Rain. (mm)	February Temp. (°C)	February Rain. (mm)
MFBR	15.1	197	17.5	75	18.3	114	17.6	115	15	241	14.3	182
HAL	9.4	62	11.4	44	12	69	11	80	8.9	168	8.2	146
HAL1	14	62	16	27	17	36.3	16	45.3	14	113	12.7	93.3
HAL2	15.8	62.4	17.7	29	18.5	34.8	17.9	43.9	15.5	102	14.6	85.4
HAN1	20.3	352	22	200	22.6	271	22.5	279	20.4	310	19.8	233
HANH	22.1	147	24.3	92.4	24.9	110	24.4	161	20.4	310	21.2	128
KAH	22.9	29	24.9	5.37	25.8	7.65	25.2	21.9	22.4	83.5	21.7	58.5
KAUM	21.2	340	22.9	149	23.4	236	23.1	258	21.1	270	20.4	203
KAUP	21.9	59	23.7	34.7	24.7	34.2	24.1	53	21.4	117	20.7	76.3
KEA	22.9	13.1	24.9	1.95	25.8	2.02	25.2	11.3	22.5	61.9	21.7	43.1
KEO	17.4	54	19.2	38.7	20.1	31.6	19.5	47.6	17	97	16.2	78.8
KIP	22.1	163	23.5	137	24.4	123	24.1	164	21.6	159	21	159
KUL	16.8	45	18.6	24.1	19.5	24.9	18.8	33.9	16.3	95.6	15.6	74.1
LAH	27.5	18.8	24.8	8.1	25.7	8.4	25.2	17.5	22.4	73.1	21.7	47.5
WAK	22	146	24	69.8	24.8	90.2	24.3	92.2	21.7	170	21.1	116
MISC	18.3	283	20.4	94.1	21.1	116	20.6	130	17.9	271	17.3	213
WAI	21.2	97.8	23.2	30.1	24.1	42.2	23.4	76.5	20.8	148	20.1	116
OLO	23	8	24.9	1.5	25.8	2.23	25.3	12.5	22.4	67	21.8	38.9

APPENDIX B: THE TOTAL NUMBER OF ADULT MOSQUITOES OF EACH SPECIES COLLECTED ACROSS ALL SITES AT A GIVEN SAMPLING PERIOD.

Month	AA	CQ	AV	WM	Total
April	44	6	1	0	51
June	127	4	3	0	134
August	26	0	0	5	31
October	59	2	0	1	62
December	15	1	0	0	16
February	24	1	0	3	28
Total	295	14	4	9	322

*AA=*Aedes albopictus*, CQ=*Culex quinquefasciatus*, WM=*Wyeomyia mitchellii*, AV=*Aedes vexans*.

APPENDIX C: SUM OF ADULT MOSQUITOES COLLECTED BY BG-SENTINEL TRAPS ACROSS SITES.

Sites	AA	CQ	AV	WM	Sum
Hana	93	4	0	0	97
Kahului	72	2	0	0	74
Kaupo	14	0	0	0	14
Kealia	1	2	3	1	7
Keokea	2	0	0	0	2
Kula	48	0	0	0	48
MFBR	11	4	1	6	22
MISC	27	0	0	2	29
OLO	27	2	0	0	29
Sum	295	14	4	9	322

*AA=Aedes albopictus, CQ=Culex quinquefasciatus, WM=Wyeomyia mitchellii, AV=Aedes vexans.

APPENDIX D: INVESTIGATING RANDOM INTERACTIONS TO ASSESS OVERALL CHANGES IN THE COMPOSITION OF THE MOSQUITO MICROBIOME

Individual level effects (i.e. Ascogregarina presence and sex) and spatial variation (i.e. Site) were assessed as random interactions in a mixed effects model to test for changes in the mosquito microbiome. The random interactions of "site x Ascogregarina presence" (p=0.99), "OTU x Ascogregarina presence x sex" (p=1), and "OTU x Ascogregarina presence x site" (p=0.22) were not significantly associated with *Ae. albopictus* microbiome.

APPENDIX E: GEOGRAPHICAL COORDINATES, AVERAGE TEMPERATURE, AND RAINFALL AT EIGHT SITES FROM WHICH ADULT AE. ALBOPICTUS MOSQUITOES WERE COLLECTED IN MAUI, HI DURING FIVE SAMPLING PERIODS.

Site	Latitude	Longitude	Elevation (m)	April temp (°C)	April rain (mm)	June temp (°C)	June rain (mm)	August temp (°C)	August rain (mm)	October temp (°C)	October rain (mm)	December temp (°C)	December rain (mm)
FBR	20.8069	-156.2804	1153	15.1	197	17.5	75	18.3	114	17.6	115	15	241
HAN	20.7635	-155.9944	6	22.1	147	24.3	92.4	24.9	110	24.4	161	20.4	310
KAH	20.8898	-156.4757	20	22.9	29	24.9	5.37	25.8	7.65	25.2	21.9	22.4	83.5
KAU	20.6346	-156.1365	50	21.9	59	23.7	34.7	24.7	34.2	24.1	53	21.4	117
KEO	20.7058	-156.3567	844	17.4	54	19.2	38.7	20.1	31.6	19.5	47.6	17	97
KUL	20.7576	-156.321	976	16.8	45	18.6	24.1	19.5	24.9	18.8	33.9	16.3	95.6
MIS	20.8408	-156.2937	660	18.3	283	20.4	94.1	21.1	116	20.6	130	17.9	271
OLO	20.8111	-156.6211	20	23	8	24.9	1.5	25.8	2.23	25.3	12.5	22.4	67

*Average temperature and rainfall measurements were acquired from Climate Atlas of Hawaii. GPS coordinates were gathered at each collection site using Garmin eTrex touch.

APPENDIX F: NUMBER OF AE. ALBOPICTUS SPECIMENS SELECTED FOR ANALYSIS FROM THE POOL OF MOSQUITOES COLLECTED ACROSS SITES AND SAMPLING PERIODS.

Site	April, 2017	June, 2017	August, 2017	October, 2017	December, 2017	Sum
FBR	0	3	1	0	0	4
HAN	4	9	10	2	1	26
KAH	10	7	2	6	2	27
KAU	0	0	2	0	1	3
KEO	0	0	0	4	1	5
KUL	9	10	4	4	1	28
MIS	0	2	1	9	0	12
OLO	6	0	1	2	4	13
Sum	29	31	21	27	10	

*Mosquitoes that were subjected to 16s rRNA sequencing at the collection sites and months. Mosquitoes were collected every two months with BG II sentinel traps.

APPENDIX G: THE AVERAGE PROPORTION OF READS FOR EACH OTU FOUND IN AE ALBOPICTUS ADULTS SAMPLED FROM EACH SITE IN MAUI, HI OVER A ONE-YEAR PERIOD. THE AVERAGE PROPORTION OF READS FOR EACH OTU, WHICH WAS CALCULATED FOR INDIVIDUAL MOSQUITOES AT EACH SAMPLING SITE.

OTUs	FBR	HAN	KAH	KAU	KEO	KUL	MIS	OLO
wAlbA	4.7E-01	7.9E-01	7.6E-01	1.9E-01	4.5E-01	7.2E-01	6.6E-01	6.6E-01
wAlbB	5.9E-02	7.5E-02	1.8E-01	2.2E-01	2.9E-01	1.6E-01	1.2E-01	2.4E-01
Asaia	2.1E-01	2.0E-02	4.8E-02	2.1E-04	0.0E+00	3.2E-02	8.3E-02	3.2E-03
Serratia	4.4E-03	2.4E-03	0.0E+00	3.3E-01	0.0E+00	1.4E-03	0.0E+00	0.0E+00
E1	3.3E-03	5.9E-02	1.9E-05	2.5E-01	0.0E+00	5.0E-02	1.2E-03	0.0E+00
E2	3.9E-03	6.8E-03	1.3E-04	0.0E+00	1.4E-01	3.6E-02	0.0E+00	0.0E+00
E3	6.3E-04	2.7E-02	0.0E+00	4.7E-03	0.0E+00	1.4E-04	6.4E-02	7.8E-02
Burkholderia	2.1E-01	1.2E-03	4.4E-03	0.0E+00	0.0E+00	3.9E-03	7.9E-03	0.0E+00
E4	0.0E+00	4.6E-04	2.9E-04	0.0E+00	3.8E-02	1.0E-03	2.3E-03	4.8E-03
Rhizobiales	0.0E+00	0.0E+00	1.2E-04	0.0E+00	0.0E+00	4.0E-05	1.4E-02	2.4E-03
Bradyrhizobium	1.8E-03	1.4E-03	2.6E-04	5.3E-04	5.8E-02	1.1E-03	2.6E-02	0.0E+00
Pseudomonas	3.2E-03	1.9E-03	3.8E-05	5.3E-03	0.0E+00	7.6E-05	4.2E-03	7.8E-04
Streptomyces	0.0E+00	4.4E-03	2.2E-04	0.0E+00	2.5E-02	1.2E-05	1.2E-02	1.1E-04
Staphylococcus	0.0E+00	4.9E-03	1.9E-04	0.0E+00	0.0E+00	1.7E-04	9.2E-05	3.1E-03
Stenotrophomonas	8.8E-03	2.4E-04	4.2E-05	0.0E+00	0.0E+00	1.3E-05	3.7E-04	2.2E-03
Methylobacterium	2.1E-03	9.1E-04	0.0E+00	0.0E+00	0.0E+00	9.3E-06	5.9E-03	0.0E+00
Rhizobium	0.0E+00	1.2E-04	2.0E-04	0.0E+00	0.0E+00	6.3E-05	3.1E-04	9.7E-05
Sphingomonas	3.0E-02	4.0E-06	1.3E-04	0.0E+00	0.0E+00	3.2E-04	4.3E-04	0.0E+00

APPENDIX H: THE PERCENTAGE OF EACH OTU FOUND IN AE. ALBOPICTUS ADULTS SAMPLED FROM EACH SITE ON THE ISLAND OF MAUI OVER A ONE-YEAR PERIOD.

OTUs	FBR	HAN	KAH	KAU	KEO	KU	MIS	OLO
wAlbA	100	100	100	100	100	93	100	100
wAlbB	50	69	85	100	100	86	82	85
Asaia	25	27	41	67	0	43	45	31
Serratia	50	12	0	33	0	11	0	0
Enterobacteriaceae 1	25	23	4	33	0	18	0	0
Enterobacteriaceae 2	25	19	7	0	20	11	0	0
Enterobacteriaceae 3	25	23	0	33	0	7	0	15
Burkholderia	75	8	56	0	0	29	18	0
Enterobacteriaceae 4	0	19	22	0	20	25	27	38
Rhizobiales	0	0	11	0	0	7	27	31
Bradyrhizobium	25	15	7	33	60	7	36	0
Pseudomonas	25	15	7	33	0	4	9	8
Streptomyces	0	8	7	0	40	4	55	15
Staphylococcus	0	4	7	0	0	4	9	8
Stenotrophomonas	25	15	11	0	0	7	9	8
Methylobacterium	25	8	0	0	0	4	36	0
Rhizobium	0	12	11	0	0	11	18	8
Sphingomonas	25	4	11	0	0	7	18	0

APPENDIX I: THE PERCENTAGE OF INDIVIDUAL AE. ALBOPICTUS ADULTS THAT WERE POSITIVE FOR ASCOGREGARINA TAIWANENSIS ACROSS EACH SITE.

Site	*Ascogregarina taiwanensis* (% positive)
FBR	100
HAN	77
KAH	93
KAU	33
KEO	80
KUL	75
MIS	92
OLO	100

APPENDIX J: SITES ACROSS MĀNOA VALLEY ON OAHU OVER A 6-MONTH PERIOD IN 2019 AND THEIR CHARACTERISTICS FOR OBJECTIVE 3

Site Number	Site Name	Date Samples Collected	Latitude	Longitude	Site Descriptors	Proximal to Human Habitation
1	Biomedical Sciences Building, UH	6/20/19	21.303938	-157.813905	Near a recycling center. Many artificial containers present (recycling bins)	Y
2	Pacific Biosciences Research Center, UH	7/26/19	21.302957	-157.813492	Near a research building with lots of artificial containers (potted plants)	Y
3	Sherman Courtyard, UH	8/5/19	21.302085	-157.815026	University building with a courtyard containing native vegetation. *Aedes albopictus* commonly captured.	Y
4	St. John Courtyard, UH	8/6/19	21.301488	-157.814902	Courtyard with native vegetation. *Aedes albopictus* commonly captured.	Y
5	Hale Laulima Dorms, UH	8/8/19	21.301162	-157.812985	Behind dorm building near the Mānoa stream. Dense vegetation with natural oviposition sites noted.	Y
6	Hale Kuahine Dorms, UH	8/8/19	21.300311	-157.813453	Behind dorm building near the Mānoa stream. Dense vegetation with artificial containers (potted plants) and natural oviposition sites noted.	Y

7	Ka Papa Lo‘i O Kānewai, UH	9/10/19	21.295216	-157.813113	Near Mānoa stream and planted Lo‘i Kalo. Dense vegetation with natural containers.	Y
8	Kamakakuokalani Center for Hawaiian Studies, UH	9/10/19	21.296441	-157.813533	Near Mānoa stream and planted Lo‘i Kalo. Dense vegetion with natural containers.	Y
9	Schidler College of Business, UH	9/24/19	21.300787	-157.820217	Near heavily landscaped building, little to no natural oviposition sites noted. Artificial containers noted (potted plants).	Y
10	Lyon Arboretum, site 1	10/29/19	21.3382	-157.80206	Near a visiting center at Lyon Arboretum botanical garden. Many natural oviposition sites.	Y
11	Lyon Arboretum, site 2	10/29/19	21.33408	-157.80305	Along trail at at Lyon Arboretum botanical garden. Natural and artificial oviposition sites.	Y
12	Lyon Arboretum, site 3	10/29/19	21.33477	-157.80229	Along trail at at Lyon Arboretum botanical garden. Natural and artificial oviposition sites.	Y
13	Lyon Arboretum, site 4	11/5/19	21.3349	-157.80301	Approximately 30 meters from trail, dense native vegetation, and natural oviposition sites.	N
14	Lyon Arboretum, site 5	11/5/19	21.33486	-157.80344	Approximately 30 meters from trail, dense native vegetation, and natural oviposition sites.	N
15	University of Hawaii Children’s Center at Mānoa, UH	12/9/19	21.297246	-157.82284	Near a children’s center with natural and artificial oviposition sites. Pet chickens approximately 5 meters from site.	Y

16	Lyon Arboretum, site 6	12/10/19	21.3373	-157.8044	Approximately 30 meters from trail, dense native vegetation, and natural oviposition sites.	N
17	Lyon Arboretum, site 7	12/10/19	21.33828	-157.80513	Approximately 30 meters from trail, dense native vegetation, and natural oviposition sites.	N
18	Lyon Arboretum, site 8	12/10/19	21.33887	-157.80597	Approximately 30 meters from trail, dense native vegetation, and natural oviposition sites.	N
19	Lyon Arboretum, site 9	12/10/19	21.33933	-157.80635	Approximately 30 meters from trail, dense native vegetation, and natural oviposition sites.	N
20	Magoon Research Station, UH	12/11/19	21.30645	-157.80906	Near a field research center with natural and artificial oviposition sites. Surrounded by housing and a school.	Y

APPENDIX K: CO-OCCURRING OTUS BETWEEN ENVIRONMENTAL SAMPLES AND MOSQUITO TISSUES. SHADED BOXES INDICATE

OTUs	Crop/ Plant	Crop/ Water	Crop/ Soil	Midgut/ Plant	Midgut/ Water	Midgut/ Soil	MT/ Plant	MT/ Water	MT/ Soil	Ovary/ Plant	Ovary/ Water	Ovary/ Soil
Acinetobacter												
Asaia												
Bradyrhizobium												
ERWIN1												
Ralstonia												
Rickettsiella												

APPENDIX L: THE AVERAGE PROPORTION OF READS FOR EACH OTU FOUND IN AE ALBOPICTUS TISSUES (CROP, MIDGUT, MALPIGHIAN TUBULE, AND OVARIES)

OTU	Crop	Midgut	Malpighian tubule	Ovary
wAlbA	1.9E-01	2.1E-01	3.1E-01	4.1E-01
wAlbB	5.4E-02	3.3E-02	2.2E-01	4.4E-01
Ralstonia	3.6E-01	3.8E-01	2.4E-01	7.0E-02
Burkholderia	2.8E-01	2.3E-01	1.9E-01	5.0E-02
ERWIN1	1.4E-02	0.0E+00	3.1E-04	1.8E-04
Asaia	4.5E-02	5.9E-02	4.3E-05	6.0E-05
Rickettsiella	3.8E-04	5.0E-04	7.0E-05	1.7E-04
Bradyrhizobium	3.0E-02	4.1E-02	3.0E-03	2.3E-03
CAULO1	9.8E-03	1.3E-02	1.2E-03	1.1E-03
CHITI1	7.4E-03	1.1E-02	1.8E-03	0.0E+00
Mesorhizobium	8.2E-03	1.1E-02	6.0E-03	0.0E+00
Sphingomonas	1.1E-03	1.4E-04	2.5E-03	0.0E+00
ACIDO1	1.9E-03	0.0E+00	0.0E+00	0.0E+00
Acidibacter	1.8E-03	3.5E-03	9.2E-04	2.0E-04
Vibrionimonas	0.0E+00	1.5E-02	2.6E-03	0.0E+00
HALO1	0.0E+00	0.0E+00	1.6E-02	2.9E-02
Xanthobacter	0.0E+00	0.0E+00	4.1E-03	0.0E+00
Acinetobacter	0.0E+00	0.0E+00	0.0E+00	6.4E-04
PLAN1	0.0E+00	0.0E+00	0.0E+00	1.1E-05
Cloacibacterium	0.0E+00	0.0E+00	0.0E+00	1.7E-04

APPENDIX M: THE PERCENTAGE OF EACH OTU FOUND IN AE. ALBOPICTUS MOSQUITO TISSUES

OTU	Crop	Midgut	Malpighian tubule	Ovary
wAlbA	95	95	100	100
wAlbB	95	90	100	100
Ralstonia	90	100	95	95
Burkholderia	95	80	100	79
ERWIN1	30	0	55	53
Asaia	45	35	30	37
Rickettsiella	30	30	30	68
Bradyrhizobium	70	65	70	37
CAULO1	45	30	60	21
CHITI1	25	30	35	0
Mesorhizobium	50	50	70	0
Sphingomonas	30	25	30	0
ACIDO1	35	0	0	0
Acidibacter	45	45	55	37
Vibrionimonas	0	30	40	0
HALO1	0	0	30	21
Xanthobacter	0	0	25	0
Acinetobacter	0	0	0	26
PLAN1	0	0	0	21
Cloacibacterium	0	0	0	21

APPENDIX N: CONDITIONAL MODES ACROSS MOSQUITO TISSUE TYPES

OTU	Crop	Midgut	Malpighian Tubule	Ovary
wAlbA	1.56	1.56	0.49	2.86
wAlbB	1.56	1.57	0.84	4.44
Ralstonia	1.56	1.56	-0.23	-0.10
Burkholderia	1.57	1.57	0.44	-0.46
ERWIN1	1.64	2.01	0.47	2.13
Asaia	1.59	1.59	-2.32	-0.40
Rickettsiella	1.62	1.61	-1.30	1.67
Bradyrhizobium	1.57	1.57	-0.56	-1.15
CAULO1	1.59	1.60	-0.16	-0.90
CHITI1	1.65	1.65	0.24	-3.84
Mesorhizobium	1.62	1.62	1.50	-4.22
Sphingomonas	1.66	1.67	0.34	-3.09
ACIDO1	1.86	2.42	-2.00	-1.42
Acidibacter	1.60	1.59	-0.47	-0.11
Vibrionimonas	2.15	1.71	1.65	-3.08
HALO1	2.15	2.25	1.41	1.88
Xanthobacter	2.37	2.49	2.68	-1.62
Acinetobacter	2.31	2.44	-2.15	3.56
Planctomycetes	2.39	2.55	-1.80	2.50
Cloacibacterium	2.38	2.48	-1.90	2.84

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