The Dissertation Committee for Miguel Arturo Saldaña Certifies that this is the approved version of the following dissertation:

Exploiting the Conflict Within: Examining Tripartite Interactions for Development of Next Generation Mosquito Microbiome Vector Control Strategies.

Committee:

Scott C. Weaver, PhD, Supervisor

Grant L. Hughes, PhD, Co-Supervisor

Alfredo G. Torres, PhD

Mariano A. Garcia-Blanco, MD, PhD

Nancy A. Moran, PhD

Dean, Graduate School

Exploiting the Conflict Within: Examining Tripartite Interactions for Development of Next Generation Mosquito Microbiome Vector Control Strategies.

by

Miguel Arturo Saldaña, BS

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Dedication

I dedicate this work to my wife and sons who patiently supported me through my training as well as the years leading up to my admission into this program and for always believing in me. To my Father and Mother who inspired me to chase after my dreams. And to all others who supported my family and me over the years.

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Exploiting the Conflict Within: Examining Tripartite Interactions for Development of Next Generation Mosquito Microbiome Vector Control Strategies.

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Miguel Arturo Saldaña, PhD

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Supervisor: Grant L. Hughes, PhD

Mosquitoes harbor various microbes that profoundly influence many aspects of their biology, including vector competence. Given their intimate association, these microbes have established a wide range of strategies aiding them in their transmission, either horizontally or vertically, making them highly attractive for applied vector control approaches to prevent the spread of arthropod-borne disease. The mosquito microbiome responds to environmental and host cues as well as microbial interactions and diet. This body of work focuses on a number of aspects import for the development of a robust microbial driven control stratagem. Next generation sequencing of the 16S ribosomal RNA gene was utilized to examine the microbiome in each of the objectives presented here. The first objective explored how different sugar types influence the microbiome of *Aedes aegypti* resulted in two main conclusions. First, mosquitoes that are reared in separarte environments had distinct microbiomes. The second finding was that, although sugar type only impatcted the overall microbial community structure in the New Orleans mosquito line from Liverpool, the Galveston mosquito line from Galveston and the NO line from Liverpool both experienced altered responses to each sugar by specific bacterial taxa. The second objective characterized the relationship between the microbiome and Zika virus (ZIKV) in both lab reared and field collected *Ae. aegypti* mosquitoes. Here bacterial

representatives of the *Acetobacteraceae* and *Enterobacteriaceae* families were correlated to ZIKV infection. The influence of these bacteria was found to be independent of mosquito immunity. Additionally, mosquitoes exposed to ZIKV had increased levels of these *Acetobacteraceae* and *Enterobacteriaceae* bacteria. These results suggest that ZIKV infection were both mosquito and viral strain specific. The third objective, which examines host small RNA interplay between mosquitoes and ZIKV infection, found that infection lead to dramatic increases in short interfering RNAs (siRNAs) and PIWI-interacting RNAs (piRNAs). Additionally, 17 host miRNAs had altered levels across multiple time points. Finally, the mosquito RNAi response to ZIKV targeted the NS5 region, while ZIKV in response produced virus-derived piRNA-like small RNAs (vipRNAs). Together, these results establish the foundations for developing a microbial based control strategy, in which bacteria could be engineered to deliver RNAi-stimulating RNAs in mosquito hosts to prevent the spread of arboviruses like ZIKV.

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AAB	Acetic Acid Bacteria
Ae.	Aedes
AeDNV	Aedes Densovirus
AgDNV	Anopheles gambia Densovirus
amiRNA	Artificial microRNA
An.	Anopheles
ANCOM	Analysis of Composition of Microbiome
CFU	Colony Forming Unit
CHIKV	Chikungunya virus
CI	Cytoplasmic Incompatibility
Cx.	Culex
DENV	Dengue viurs
DNV	Densovirus
DPI	Days Post Infection
dsRNA	Double Stranded RNA
EEEV	Eastern Equine Encephalitis virus
FC	Fold Change
FFA	Focus Forming Assay
FFU	Focus Forming Unit
gDNA	Genomic DNA
HTS	High Throughput Sequencing
IT	Intrathoracically microinjected
IIT	Incompatible Insect Technique
ISV	Insect-Specific viruses
JEV	Japanese Encephalitis virus
LACV	La Crosse virus
lhRNA	Long-hairpin RNA
LSTM	Liverpool School of Tropical Medicine

miRNA	microRNA
NGS	Next Generation Sequencing
nMDS	Non-metric Multidimensional Scaling
ONNV	O'nyong nyong virus
OTU	Operational Taxonomic Unit
PAGE	Polyacrylamide Gel Electrophoresis
PCV	Palm Creek virus
piRNA	PIWI-Interacting RNA
RGV	Rio Grande Valley
RISC	RNA Induced Silencing Complex
RNAi	RNA Interference
ROS	Reactive Oxygen Species
rRNA	Ribosomal RNA
RVFV	Rift Valley Fever virus
sfRNA	Subgenomic Flavivirus RNA
SFV	Semliki Forest virus
SGHV	Salivary Gland Hypertrophy virus
shRNA	Short-hairpin RNA
SINV	Sindbis virus
siRNA	Small Interfering RNA
viRNA	Virus-derived short Interfering RNA
UTMB	Unv. of Texas Medical Branch
VBD	Vector-borne Diseases
WNV	West Nile virus
YFV	Yellow fever virus
ZIKV	Zika virus

INTRODUCTION: MOSQUITO BIOLOGY & ARBOVIRUSES

Aedes aegypti, also known as the yellow fever mosquito, is a member of the fly family *Culicidae* and undergoes complete metamorphosis, which includes 4 distinct stages beginning with an egg, followed by the larvae, pupae and ending with the adult stage. Development usually occurs over a one-week period, beginning in an aquatic environment (egg through to pupae) and ending with the emergence of the adult into the final terrestrial environment. In rural environments, *Aedes* mosquitoes breed in natural reservoirs of water such as tree hollows or leaf matter, giving them the moniker of tree hole breeders. In urban environments the mosquitoes have taken to breeding in artificial containers including bottles and flower pots (Lima *et al.*, 2016). Adult mosquito lifespan can range from one week up to one month (Nelson, 1986), while some species located in cooler or dryer climates are able to over winter (Watts *et al.*, 1974; Lima *et al.*, 2016). One example of this is the ability for *Aedes* mosquito eggs to dry out for an extended period of time, still being viable to later become hydrated and hatch (Hawley *et al.*, 1989). Another method available to mosquitoes in urban settings is to enter homes and avoid the cold altogether. This is highly advantageous to urban or domesticated mosquitoes as it keeps them in close proximity to a potential blood supply. *Aedes* mosquitoes are hematophagous and anautogenous insects, meaning that a blood meal is needed to complete the gonotrophic cycle to produce eggs (Gulia-Nuss *et al.*, 2012). *Ae. aegypti* females often search out dark areas to rest in between blood meals, often times these locations are inside homes. In some regions it is suggested that these domesticated mosquitoes have adapted to strictly blood feeding (Edman *et al.*, 1992).

Hematophagy is an important trait as mosquitoes are known vectors for a variety of pathogens ranging from viruses to parasites. *Ae. aegypti* have been linked to the transmission of a number of emerging and re-emerging arboviruses. These viruses can be divided into two main viral families, the *Alphaviruses* and *Flaviviruses,* both of which are small single stranded positive sense RNA viruses. *Aedes* mosquitoes typically transmit arthritis causing alphaviruses, which include Chikungunya virus (CHIKV), Mayaro virus (MAYV), O'nyong nyong virus (ONNV), Ross River virus (RRV), and Semliki forest virus (SFV). The second group transmitted by *Aedes* are hemorrhagic disease causing flaviviruses including Yellow Fever virus (YFV), dengue virus (DENV), and Zika virus (ZIKV). Both of these viral groups typical have an amplifying vertebrate host that completes the enzootic life cycle of the virus, while humans are dead-end hosts in this cycle; however, in epizootic lifecycles these viruses can directly follow a mosquito-human cycle in which humans serve as the amplifying host. The emergence and spread of these arboviruses highlight the importance of vector control strategies and more importantly their shortcomings. Current vector control strategies are limited to proximity of application, chemical half-life and developed resistance, further demonstrating a need for the development of new strategies. Here the current state of microbial-based vector control strategies is discussed and focused on several aspects of the relationship between mosquitoes, their microbiota and viruses, which can be utilized for future development of a microbial based strategy.

Chapter 1 Microbial control of Mosquito Borne Viruses[1]

Vector-borne diseases (VBD) are responsible for inordinate mortality, morbidity and economic loss worldwide. One of the most important groups of pathogens transmitting vectors are the mosquitoes, including species within the *Anopheles*, *Aedes* and *Culex* genera. Particularly well studied are the *Anopheles* mosquitoes that vector *Plasmodium* parasites that cause malaria in humans. While five *Plasmodium* parasites cause malaria, *Plasmodium falciparum* is the major cause of this disease in sub-Saharan Africa (Snow *et al.*, 2005). *Aedes* mosquitoes are notorious for vectoring arthropod borne viruses (arboviruses) including flaviviruses such as dengue virus (DENV), Yellow fever virus (YFV) and Zika virus (ZIKV), and also the Alphavirus, Chikungunya virus (CHIKV) (Bhatt *et al.*, 2013; Weaver & Lecuit, 2015; Weaver *et al.*, 2016). *Culex* mosquitoes are known vectors of West Nile virus (WNV) and other encephalitic viruses, as well as filarial nematodes. Other than mosquitoes, Phlebotominae and Simuliidae flies are responsible for transmitting pathogens that cause Leishmaniasis, Onchocerciasis, as well as other neglected tropical diseases. In Africa, several species of tsetse flies vector Trypanosomes that cause sleeping sickness in humans and nagana in livestock. Further vectors include Triatomine bugs that transmit Trypanosomes that cause Chagas disease, which infects an estimated 6 million people in Latin America (Bern, 2015). Ticks also transmit a variety of pathogens including viral, bacterial and protozoan parasites (Dantas-Torres *et al.*, 2012). While traditional and contemporary control strategies have made great progress to control malaria and other neglected tropical diseases, the incidence of other diseases has been on the rise. Current disease prevention strategies often rely on vector control as effective vaccines are not available for many pathogens, however vector control strategies are

[1] The work mentioned in this chapter is based on the work published **Saldaña MA**, Hegde S, Hughes GL. Microbial Control of Arthropod-Borne Disease. Mem Inst Oswaldo Cruz. 2017 Feb; 112(2):81-93. PMID: 28177042. The Creative Commons License can be accessed at http://creativecommons.org/publicdomain/zero/1.0/

becoming ineffective, mainly due to insecticide resistance emerging in many vectors (Naqqash *et al.*, 2016; Ranson & Lissenden, 2016). Taken together, novel strategies for control of VBD are urgently required. The current global ZIKV pandemic, and the reemergence of YFV in Africa and *Leishmania* in the Middle East stress this need for novel control tools against emerging and re-emerging pathogens (Al-Salem *et al.*, 2016; Barrett, 2016; Weaver *et al.*, 2016). To this end, microbial-based intervention strategies are gaining considerable traction as a novel means to control VBD. In this chapter we highlight the recent advances in the use of symbionts to suppress pathogens in their vectors by drawing upon examples of viral, bacterial and fungal symbiosis in various vector species. Most studies have focused on mosquito vectors but where possible we include examples from other vector systems.

THE VECTOR MICROBIOME - The advent of High Throughput Sequencing (HTS) technologies has expanded our understanding of the composition of the microbiome of many vector species. The microbiome is composed of viruses, bacteria, fungi and protozoa, however pathogens that vectors transmit can also be considered as constituents of the microbiome. Microbial association with the host can be facultative or obligate, and the nature of these host-microbe interactions, which range across a spectrum from parasitic to mutualistic, is likely fluid and depends on factors such as the host and environment (Casadevall *et al.*, 2011). Microbes can have an intracellular or extracellular lifestyle, and possibly transition between both. Microbiota can also preferentially reside in specific host organs and tissue including the midgut (the lumen or gut epithelia), fat body, salivary glands, ovaries and testes (Sharma *et al.*, 2014; Segata *et al.*, 2016; Tchioffo *et al.*, 2016). In several of these tissues, the microbe has the opportunity to directly interact with invading pathogens.

Our most comprehensive understanding of vector microbiomes is derived from mosquitoes. Studies utilizing HTS have revealed that the microbiome is often dominated by relatively few taxa, can be highly variable, and that this variation is influenced by factors such as host life stage, host sex, the sampling technique, and the biotic and abiotic environment (Boissière *et al.*, 2012; Osei-Poku *et al.*, 2012; Coon *et al.*, 2014; Gimonneau *et al.*, 2014; Duguma *et al.*, 2015; Buck *et al.*, 2016; Segata *et al.*, 2016). HTS techniques are currently most effective in examining the bacterial microbiome, and such work suggests mosquitoes have a microbiota comprised of bacteria within the phyla *Proteobacteria*, *Bacteriodetes* and *Actinobacteria*, encompassing taxa such as *Serratia*, *Pseudomonas*, *Aeromonas*, *Elizabethkingia*, *Enterobacter*, and *Acintobacter* (Boissière *et al.*, 2012; Osei-Poku *et al.*, 2012; Coon *et al.*, 2014; Gimonneau *et al.*, 2014; Hughes *et al.*, 2014a; Duguma *et al.*, 2015; Buck *et al.*, 2016; David *et al.*, 2016; Segata *et al.*, 2016). Similar to mosquitoes, ticks have been found to have diverse and complex microbiomes, with the microbial composition influenced by life history traits and diet (Menchaca *et al.*, 2013). The microbiome of lone star tick, *Amblyomma americanum*, is composed of the pathogens *Anoplasma* and *Ehrlichia* as well as other symbiotic bacteria within the phyla *Proteobacteria*, *Bacteroidetes* and *Firmicutes* (Jasinskas *et al.*, 2007; Fryxell & DeBruyn, 2016). Microbiome analysis of the Rocky mountain wood tick, *Dermacentor andersoni* identified four prominent genera of bacteria: *Rickettsia*, *Francisella*, *Arsenophonus* and *Acinetobacter* (Clayton *et al.*, 2015). In tsetse flies, three vertically transmitted bacterial symbionts, *Wigglesworthia*, *Sodalis*, and *Wolbachia* are often present in the host, in addition to other environmentally acquired commensal bacteria (Wang *et al.*, 2013b).

There are few studies investigating the fungal microbiome (mycobiome) of vector species. Most approaches that do explore the diversity of fungal microbes in insects exploit culture-based methods (Ignatova *et al.*, 1996; Marti *et al.*, 2006; Gusmão *et al.*, 2010). A yeast strain, *Wickerhamomyces anomalus,* was found in both the midgut and reproductive

system of the Asian malaria vector, *Anopheles stephensi* (Ricci *et al.*, 2011a), and six different fungal species have been found in the midgut of sandfly vectors (Akhoundi *et al.*, 2012). However, recently, HTS was used to examine the mycobiome of *Aedes triseriatus* and *Aedes japonicus* (Muturi *et al.*, 2016). This study found twenty-one distinct fungal OTUs, 15 of which were shared between *Ae. triseriatus and Ae. japonicus* (Muturi *et al.*, 2016). The majority of fungal taxa in these *Aedes* species were from the *Ascomycota* phylum (Muturi *et al.*, 2016). Similarly, the *Ae. albopictus* mycobiome is dominated by fungi within the *Ascomycota* in addition to other taxa within phylum *Basidiomycota* (Muturi *et al.*, 2016). While the role of the mycobiome in regulating vector competence is poorly understood, it is likely that fungi and yeast can have a similar impact on pathogen transmission as bacteria, as fungi produce antimicrobial molecules and influence host immunity (Lemaitre *et al.*, 1996; Wang *et al.*, 2015; Angleró-Rodríguez *et al.*, 2016; Martin *et al.*, 2016). For instance, it was recently reported that *Penicillium chrysogenum* increases the intensity of *Plasmodium* infection in *Anopheles* mosquitoes by suppressing mosquito immunity (Angleró-Rodríguez *et al.*, 2016).

Characterization of the viral microbiome (virome) of disease vectors is now also gaining attention. Metagenomic sequencing of mosquitoes revealed the presence of several species of plant, animal and bacterial viruses in the mosquito virome (Ng *et al.*, 2011; Chandler *et al.*, 2015). Similar studies in ticks also identified several viral families, including previously unknown viruses (Tokarz *et al.*, 2014; Xia *et al.*, 2015; Sakamoto *et al.*, 2016). The effect on the host of many of these viruses is yet to be elucidated. In contrast, we know that tsetse flies harbor a salivary gland hypertrophy virus (SGHV), which is a rod-shaped, enveloped DNA virus that is transmitted both horizontally and vertically, and can become pathogenic, causing hypertrophy of the salivary glands and reduced fecundity and lifespan (Wang *et al.*, 2013a). Interestingly, it appears that there is an interaction of

SGHV with microbial symbionts residing in the fly, as aposymbiotic flies have reduced viral loads (Boucias *et al.*, 2013; Wang *et al.*, 2013a).

Complex host-microbe interactions dictate microbiome and host homeostasis of arthropods. While the factors that shape the composition of the microbiome are still under investigation in most systems, it is clear that environmental conditions (Wang *et al.*, 2011; Zouache *et al.*, 2011; Minard *et al.*, 2013), and host genetics (Kumar *et al.*, 2010; Oliveira *et al.*, 2011; Stathopoulos *et al.*, 2014; Soares *et al.*, 2015; Pang *et al.*, 2016) are important. For instance, silencing of an antimicrobial peptide in *Triatoma infestans* elevated bacterial load in the midgut which subsequently reduces *Trypanosoma cruzi* parasites, indicating that host control of the microbiome can influence pathogen dynamics (Buarque *et al.*, 2016). Bacterial genetics also appears to be an important determinant of gut colonization (Maltz *et al.*, 2012; Pei *et al.*, 2015), however like much of the work examining bacterial genetic factors that influence persistence in the mammalian gut, this area of study is in its infancy in arthropods. While we have a limited understanding of the factors that regulate homeostasis in vectors, insights can be drawn from model insects where these processes have been examined in (Buchon *et al.*, 2013; Erkosar *et al.*, 2013; Broderick Nichole A., 2016). In insects, microbial interactions are known to influence many diverse phenotypes and processes including host nutrition, reproduction, immunity, behavior, survival and evolution (Engel & Moran, 2013; Lewis & Lizé, 2015; Shropshire & Bordenstein, 2016; van Tol & Dimopoulos, 2016). In arthropod vectors, these phenotypes can have important implications for vectorial capacity. Additionally, members of the microbiome can themselves modulate vector competence for a variety of pathogens, either by direct interactions with the pathogen or indirectly mediated by the host (Dennison *et al.*, 2014; Hegde *et al.*, 2015). While the influence of the microbiome on vector competence is likely multifaceted and complex, interplay between the microbiota and host immunity is one process that can alter pathogen levels (Xi *et al.*, 2008; Dong *et al.*, 2009; Carissimo *et al.*,

2015). Given these interactions, it is unsurprising that these interactions can also be reciprocated, whereby pathogen infection, which stimulates host immunity, can alter the microbiome (Xi *et al.*, 2008; Ramirez *et al.*, 2012; Zouache *et al.*, 2012; Vieira *et al.*, 2015; Zink *et al.*, 2015; Muturi *et al.*, 2016). This highlights the intricate dynamism between the host and the microbiome, which in part, is shaped by host immunity. From an applied perspective, these microbe-mediated alterations in vector competence can be harnessed for novel microbial pathogen control strategies.

INNATE ANTI-PATHOGEN ACTIVITY OF MICROBES - *Wolbachia* - The most extensively developed microbial strategy to alter the vector competence of mosquitoes utilizes *Wolbachia*. *Wolbachia* is a common bacterial endosymbiont that infects approximately 60% of insects (Hilgenboecker *et al.*, 2008). It has been extensively studied for its ability to manipulate the reproduction of its host, which enables the bacterium to spread through insect populations (Werren *et al.*, 2008). Cytoplasmic incompatibility (CI) is one of the most widespread reproductive mechanisms *Wolbachia* employs. CI occurs when an infected male mates with a female that is uninfected, or infected with an incompatible strain of *Wolbachia*. These crosses result in embryonic lethality and provide a fitness advantage to the infected female counterparts in the population, facilitating *Wolbachia's* spread within insect populations (Werren *et al.*, 2008). *Wolbachia*-mediated CI is being exploited as a population suppression tool termed incompatible insect technique (IIT) (reviewed in Bourtzis *et al.*, 2014), and has been deployed to suppress *Aedes* mosquito populations (O'Connor *et al.*, 2012). However, after it became evident that the antiviral properties of *Wolbachia,* which were first discovered in *Drosophila* (Hedges *et al.*, 2008; Teixeira *et al.*, 2008) also occurred in mosquitoes against a broad range of pathogens (Kambris *et al.*, 2009; Moreira *et al.*, 2009; Hughes *et al.*, 2011b), the use of this bacteria for population replacement control strategies has been explored with vigor. The ability of the bacterium to confer pathogen interference, and to rapidly invade populations due to a

high vertical transmission rate and the induction of CI, make *Wolbachia* an attractive agent for applied control.

Wolbachia can interfere with the development of diverse pathogens transmitted by mosquitoes. The antipathogen phenotype is particularly noticeable when a strain of *Wolbachia* is artificially transferred (transinfected) into a vector creating a novel strain-host combination (Hughes & Rasgon, 2014). Most attention has focused on *Ae. aegypti*, which is generally thought to be naturally uninfected by *Wolbachia*, however, intriguingly, an infection was recently reported in mosquitoes collected in Florida, USA (Coon *et al.*, 2016). Two strains of *Wolbachia* were found in these mosquitoes, which were phylogenetically related to the *w*AlbA and *w*AlbB strains in *Ae. albopictus* (Coon *et al.*, 2016). Transinfected *Ae. aegypti* have reduced vector competence to several important arboviruses such as DENV (Moreira *et al.*, 2009; Walker *et al.*, 2011; Joubert *et al.*, 2016), YFV (Hurk *et al.*, 2012), CHIKV (Moreira *et al.*, 2009; Aliota *et al.*, 2016a) and ZIKV (Aliota *et al.*, 2016b; Dutra *et al.*, 2016). *Wolbachia* infected *Ae. aegypti* are also less competent vectors for filarial nematodes (Kambris *et al.*, 2009) and *Plasmodium* parasites (Moreira *et al.*, 2009). In addition to arbovirus control approaches in *Aedes* mosquitoes, *Wolbachia*-based strategies are also under investigation to inhibit Japanese encephalitis virus (JEV) vectored by *Culex tritaeniorhynchus* (Jeffries & Walker, 2015).

Antiviral activity is also seen when novel strains are transinfected into *Ae. albopictus* (Blagrove *et al.*, 2012), which is naturally infected with two strains of *Wolbachia*, *w*AlbA and *w*AlbB. Here, these resident strains were removed by antibiotic treatment before introduction of the novel *w*Mel strain from *Drosophila*. These *w*Mel-infected *Ae. albopictus* have decreased vector competence for DENV compared to an uninfected line and the naturally double infected mosquitoes (Blagrove *et al.*, 2012). The effect of natural *Wolbachia* infections on pathogen dynamics is more difficult to assess, as

uninfected individuals need to be identified, or the infection cleared with antibiotic treatment, for comparison. Antibiotic treatment can also have confounding effects such as altering the microbiome (Hughes *et al.*, 2014a) or affecting mitochondria (Ballard & Melvin, 2007). With these caveats in mind, native *Wolbachia* infections have been shown to reduce WNV in *Cx. quinquefasciatus* (Glaser & Meola, 2010) and DENV and CHIKV in *Ae. albopictus* (Mousson *et al.*, 2010, 2012), but it is important to note that these naturally infected mosquitoes are still competent vectors. Conversely, the native *Wolbachia* infection in *Culex pipiens* has been shown to exacerbate *Plasmodium* titer compared to their uninfected counterparts (Zélé F. *et al.*, 2014), and *Wolbachia* also protects the vector against the deleterious fitness effects of the parasite, thus extending host lifespan, which has implications for pathogen transmission (Zélé *et al.*, 2012).

The development of *Wolbachia* control strategies for human malaria appears more complex compared to arboviral pathogens. Aside from the propensity of *Wolbachia* to increase *Plasmodium* titer in some circumstances (Hughes *et al.*, 2012; Baton *et al.*, 2013; Murdock *et al.*, 2014), which may be an artifact due to the method of infection or artificial nature of some tripartite combinations used in laboratory studies (reviewed in Hughes *et al.*, 2014b), there are challenges with stably transinfecting *Anopheles* mosquitoes. To overcome these issues, transient infection was used to rapidly asses the effect of *Wolbachia* on *Plasmodium*, and this technique found that the *w*MelPop and *w*AlbB *Wolbachia* strains blocked *P. falciparum* (Hughes et al. 2011b). The *w*MelPop strain has also been shown to interfere with *Plasmodium berghei*, a murine malaria model (Kambris *et al.*, 2010). In groundbreaking work from Bian et al. (2013) *An. stephensi* was stably infected with the *w*AlbB strain of *Wolbachia*. These novel infections induced CI in *An. stephensi* and substantially blocked *P. falciparum* (Bian *et al.*, 2013), offering promise for the use of this bacterium in malaria control approaches. However, the infection also exerted a

considerable fitness cost on the mosquito (Bian *et al.*, 2013; Joshi *et al.*, 2014), which would need to be overcome for *Wolbachia* to spread in field populations.

Recently, natural *Wolbachia* infections in some *Anopheles* populations have been discovered (Baldini *et al.*, 2014; Buck *et al.*, 2016; Shaw *et al.*, 2016). These studies, in addition to the transinfection of *An. stephensi* (Bian *et al.*, 2013), have overturned the dogma that *Anopheles* mosquitoes were recalcitrant to *Wolbachia* infection and were naturally uninfected across their range. The native infections were shown to affect host fitness and reduce *Plasmodium* loads compared to uninfected conspecifics (Shaw *et al.*, 2016). More work is required to determine if these natural infections can be exploited for *Plasmodium* control or if the resident strains would complicate the spread of more useful transinfected strains (Jeffries & Walker, 2016). Similarly, the recently discovered natural infections in *Ae. aegypti* could have implications for implementation of *Wolbachia*-based strategies (Coon *et al.*, 2016). Other bacterial symbionts that are known to manipulate insect reproduction (Duron *et al.*, 2008), in a similar fashion to *Wolbachia* such as *Spiroplasma* (Terenius *et al.*, 2008; Segata *et al.*, 2016), and bacteria related to *Arsenophonus* (Briones *et al.*, 2008) have been found in mosquitoes, but their effect on host reproduction and vector competence remains to be elucidated.

GUT ASSOCIATED MICROBES - Bacteria that reside predominately within the midgut of vectors can have profound anti-pathogenic effects that could be exploited in novel vector control strategies. Early studies examined the interaction between microbes and pathogens in *Anopheles-Plasmodium* and *Triatomine-Trypanosome* systems (Beier *et al.*, 1994; Straif *et al.*, 1998; Eichler & Schaub, 2002). Today, most research in this area focuses on *Aedes* and *Anopheles* mosquitoes and the influence of the microbiome on arboviruses and *Plasmodium* parasites, respectively. Research that investigates the influence of gut microbes on pathogen dynamics is usually undertaken by perturbing the

microbiome by antibiotic treatment or through administration of cultured bacteria to the vector. Alternative approaches included using antibodies raised against the microbiota to manipulate the microbiome, or rearing gnotobiotic lines (Noden *et al.*, 2011; Coon *et al.*, 2014). Antibiotic treatment has been shown to increase the titer of DENV in *Ae. aegypti*, JEV in *Culex bitaeniorhynchus*, *T. cruzi* in *Rhodnius prolixus* and *Plasmodium* in *Anopheles* mosquitoes (Mourya & Soman, 1985; Xi *et al.*, 2008; Dong *et al.*, 2009; Kumar *et al.*, 2010; Rodrigues *et al.*, 2010; Castro *et al.*, 2012). These findings suggesting that the microbiota is antagonistic to invading pathogens. Re-infection of bacterial taxa into the vector enables the anti-pathogenic properties of specific microbes to be identified. Using this approach, isolates of *Enterobacter*, *Acinetobacter*, *Pantoea*, *Pseudomonas*, *Serratia* and *Elizabethkingia* have been shown to inhibit *Plasmodium* (Cirimotich *et al.*, 2011; Bahia *et al.*, 2014; Ramirez *et al.*, 2014). The *Enterobacter* Esp_Z isolate was shown to produce reactive oxygen species (ROS) that inhibited the malaria parasite (Cirimotich *et al.*, 2011), while other bacterial taxa may have distinct modes of action against *Plasmodium* (Bahia *et al.*, 2014). Intriguingly, a specific strain of *Serratia* that has enhanced motility suppresses *Plasmodium* compared to a non-motile strain, providing insights into the mechanism behind the interference phenotype and highlighting the importance of bacterial inter-strain variation on vector competence (Bando *et al.*, 2013). In other work, *Enterobacter*, *Proteus* and *Paenibacillus* species have been shown to inhibit La Crosse virus (LACV) and DENV (Joyce *et al.*, 2011; Ramirez *et al.*, 2012). Strikingly, a *Chromobacterium* isolate has both anti-*Plasmodium* and anti-viral properties, and reduces the survival of larvae and adult mosquitoes, possibly linked to the secretion of metabolites such as cyanide (Ramirez *et al.*, 2014). Secreted molecules that have anti-pathogen and entomopathogenic activity could be harnessed for novel biotechnology applications. Such products could be used against the vector or the pathogens they transmit, or alternatively, exploited as novel pharmaceuticals for use in humans or livestock.

In addition to studies on arboviruses and malaria, bacterial microbes can alter pathogens in other vector species. *Serratia*, which is a dominant component of the gut microbiome of Triatomine bugs, appears to be an important determinant of *Trypanosome* infection (Azambuja *et al.*, 2004; da Mota *et al.*, 2012). The trypanocidal activity of *Serratia* could be related to prodigiosin production, which affects the mitochondrial activity of the parasite, and the ability of this bacterium to attach to the parasite (Castro *et al.*, 2007; Genes *et al.*, 2011). Studies in sandflies imply that microbes reduce *Leishmania* parasite load (Schlein *et al.*, 1985) while tsetse flies cured of their symbionts were more susceptible to *Trypanosome* infection (Wang *et al.*, 2009; Weiss *et al.*, 2013). In ticks, both positive and negative interactions between symbionts and pathogens have been observed. *Rickettsia bellii* is negatively correlated with *Anaplasma marginale* infection, and reductions in a *Francisella* symbiont leads to a lower titer of the pathogenic *Francisella novicida* (Gall *et al.*, 2016). Perturbing the microbiome of *Ixodes scapularis* altered the peritrophic matrix of the arachnid and subsequently led to a reduction in the spirochete, *Borrelia burgdorferi* (Narasimhan *et al.*, 2014).

Pathogen enhancement mediated by microbes has also been documented in mosquitoes. Suppression of the midgut microbiota by antibiotic treatment in *Anopheles* mosquitoes decreased O'nyong nyong virus (ONNV) infections (Carissimo *et al.*, 2015), indicating that constituents of the microbiota are required for pathogen infection. Reinfection of live, but not heat-killed bacteria, into antibiotic treated mosquitoes reverted viral titers to levels comparable to untreated controls (Carissimo *et al.*, 2015). These effects are in contrast to what is observed with *Plasmodium* which increase in titer after antibiotic treatment of mosquitoes (Dong *et al.*, 2009; Kumar *et al.*, 2010; Rodrigues *et al.*, 2010). A similar pathogen enhancement effect was seen in *Ae. aegypti* re-infected with *Serratia odorifera,* which increases both DENV and CHIKV infections (Apte-Deshpande *et al.*, 2012, 2014). The ability of bacterial taxa to both enhance and suppress pathogens in insects

suggests complex interplay between the host, the microbiome and the pathogen, dictates vector competence. Furthermore, specific vector- pathogen-microbe combinations may have unique outcomes, which means intervention strategies need to be scrutinized thoroughly before implementation.

While studies examining the role of the bacterial microbiome on arthropod biology are expanding and providing insights into alternative approaches to control arthropod-borne disease, we have a very limited knowledge on the role of the virome or mycobiome on vector biology and vector competence. The yeast *W. anomalus* produces a toxin that has in vitro antiplasmodial activity (Valzano *et al.*, 2016). Studies investigating the entomopathogenic fungi *Beauveria bassiana* indicate this fungal pathogen suppresses DENV titer in *Ae. aegypti* through activation of the Toll and Jak-Stat immune pathways (Dong *et al.*, 2012). This antiviral property further supports the use of this microbe for novel microbial biopesticide applications. Recently it has become evident that mosquitoes are naturally infected with insect-specific viruses (ISV). These viruses, which are phylogenetically diverse, infect mosquitoes but do not replicate within vertebrate cells (Blitvich & Firth, 2015; Bolling *et al.*, 2015; Vasilakis & Tesh, 2015). Interestingly, it appears that ISV can suppress arboviruses in mosquitoes, likely due to a process known as superinfection exclusion (Newman *et al.*, 2011; Bolling *et al.*, 2012; Crockett *et al.*, 2012; Kenney *et al.*, 2014; Kuwata *et al.*, 2015; Hall-Mendelin *et al.*, 2016). Most studies have used in vitro systems and focused on insect-specific flaviviruses although an insect-specific alphavirus has been shown to alter Sindbis virus titer in vivo (Nasar *et al.*, 2015). These findings have raised the possibility that fungi and ISV could be used in applied control strategies but before this can be achieved, a more thorough understanding of the biology of these microbes is required. Studies should focus on examining the ecological range and infection frequency of these microbes in natural mosquito populations, understanding the

nature of their association with the host and other microbes, and investigate the mechanisms in which they are acquired and transmitted.

ENGINEERING MICROBES TO CONVEY ANTI-PATHOGEN ACTIVITY - Microbes that reside within the gut of vectors can be engineered to secrete anti-pathogen molecules, an approach known as paratransgenesis. Paratransgenic studies were initially pioneered in Triatomine bugs for control of Chagas disease (Durvasula *et al.*, 1997; Beard *et al.*, 2002). Here, the symbiotic bacterium *Rhodococcus rhodnii* was genetically manipulated to express antimicrobial peptides that were antagonistic to *T. cruzi*, the parasitic protozoan that causes Chagas disease. Expression of cecropin A eliminated or reduced the number of *T. cruzi* within *R. prolixus* (Durvasula *et al.*, 1997). Ingeniously, the copraphagic tendencies, or probing of fecal droplets, of the insect were exploited to deliver the transgenic symbiont to the vector. An artificial mimic of *R. prolixus* feces spiked with transgenic *R. rhodnii,* which was probed by nymphs, facilitated symbiont acquisition (Durvasula *et al.*, 1997). In field trials, around half of the nymphs exposed to the mimic were infected throughout their development (Durvasula *et al.*, 1999).

After these seminal studies, Beard et al. (2002) detailed the requirements for successful paratransgenic strategies. These include: that a symbiotic relationship occur between the microbe and the host; that the microbe be readily culturable and transformable; transformation should not alter the symbiotic relationship with the host, alter microbial fitness compared to wild type conspecifics or make the microbe pathogenic; that the effector gene product should be secreted to interact with the pathogen; and that there must be an efficient way to deliver the microbe into the vector population.

Paratransgenesis is also being explored in other vector species, particularly *Anopheles* mosquitoes for the control of malaria, using bacterial microbes as delivery vehicles. Earlier studies investigated engineering effector protein secretion systems from

Pantoea agglomerans, which was isolated from *Anopheles* mosquitoes (Riehle *et al.*, 2007; Bisi & Lampe, 2011). Importantly, transgenic bacteria administered to mosquitoes in sugar meals were seen to rapidly proliferate following a blood meal and had minimal impact on life history traits of the mosquito (Wang *et al.*, 2012). The secretion of several effector proteins antagonistic to *Plasmodium* using the HlyA secretion system from *P. agglomerans* was shown to significantly reduce the intensity of *P. falciparum* in the mosquito gut (Wang *et al.*, 2012). The mode of action and the targets of the anti-*Plasmodium* effector molecules has been comprehensively reviewed (Wang & Jacobs-Lorena, 2013). *Asaia* is another candidate for paratransgenic control of malaria. This bacterium is important for larval development of *Anopheles* mosquitoes, is genetically tractable, appears to be easily acquired by mosquitoes and is vertically inherited to progeny (Favia *et al.*, 2007; Chouaia *et al.*, 2012). Secretion of the effector proteins, Scorpine and the anti-Pbs21 scFv-Shiva1 toxin fusion protein, from *Asaia* reduced oocyst intensity of *P. berghei* in the midgut compared to control bacteria (Bongio & Lampe, 2015). *Elizabethkingia* is another dominant member of the mosquito microbiome that is transstadially transmitted. This bacterium has been genetically altered and reinfected into *Anopheles* and *Aedes* mosquitoes (Chen *et al.*, 2015a), however the use of this microbe in paratransgenic control approaches may need to be reconsidered since it is potentially a human pathogen (Frank *et al.*, 2013) and given its natural resistance to several antibiotics. Genomic and further epidemiological analysis may clarify if strains present in mosquitoes are the source of infection in humans (Kukutla *et al.*, 2014; Teo *et al.*, 2014; Garay *et al.*, 2016).

Paratransgenic approaches are also being developed for the control of *Trypanosomes* vectored by tsetse flies. The symbiont *Sodalis glossinidius* has been manipulated to release anti-trypanosome nanobodies (antigen binding molecules) in the fly gut (De Vooght *et al.*, 2012, 2014). Strategies have proposed to couple paratransgenic *Sodalis* with *Wolbachia* and exploit *Wolbachia's* CI-mediated drive to spread the

transgenic symbiont through the population. Modeling suggests that if *Wolbachia* induced mortality is low and the anti-trypanosome molecule is effective, the incidence of disease could be successfully reduced (Medlock *et al.*, 2013). Preliminary experiments such as the identification and culturing of microbes have been accomplished for paratransgenesis strategies in *Phlebotomus argentipes* sand flies for control of *Leishmania* (Hillesland *et al.*, 2008).

In comparison to bacterial paratransgenic approaches, there are few examples of the use of viral or fungal symbionts for paratransgenic control. While fungal paratransgenic studies are limited in medical vector species, approaches are also being investigated to control agricultural pathogens (Hughes *et al.*, 2011a). The identification of culturable fungi and yeast associated with vectors provides candidate microbes for further investigation (Ricci *et al.*, 2011a, 2011b; Martin *et al.*, 2016; Steyn *et al.*, 2016). In a subtle variation on the paratransgenic theme, the fungal insect pathogen *Metarhizium anisopliae* has been manipulated to express effector molecules to inhibit *Plasmodium* in *Anopheles* mosquitoes (Fang *et al.*, 2011). Expression of the peptide SM1, a single chain antibody, or the antimicrobial toxin scorpine, significantly reduced sporozoites in the salivary gland. Impressively, the expression of 8 repeats of SM1 and scorpine as a fusion protein reduced *Plasmodium* intensity by 98% (Fang *et al.*, 2011). *M. anisopliae* is an insect pathogen that infects mosquitoes through direct contact with the cuticle, which may enhance infection of the vector, but its pathogenic nature would likely mean that continual release of the microbe would be required.

Viral paratransgenesis research has mainly focused on Densoviruses. *Aedes* DNV (AeDNV), which can be pathogenic to the mosquito host (Ledermann et al. 2004), has been manipulated to express foreign genes (Afanasiev *et al.*, 1999). Expression of a toxin from AeDNV increased the pathogenic effects of the virus compare to wild type virus in *Ae. albopictus* (Gu *et al.*, 2010), offering promise for this strategy to be employed as a

biopesticide. An *Anopheles gambiae* DNV (AgDNV) has been characterized and used as an expression platform (Ren *et al.*, 2008; Suzuki *et al.*, 2014). Unlike AeDNV, AgDNV is not pathogenic to the mosquito host and has minimal impact on mosquito survival (Ren *et al.*, 2014). While DNVs can be used to express proteins in mosquitoes and the virus infects relevant organs in the insect to interfere with invading pathogens, there are some obstacles that need to be overcome before these viruses can be used in the field for paratransgenesis. DNVs have small genomes, which can limit the size of the inserted transgenes and they often require wild type virus for effective viral packaging. In an elegant approach, recombinant AeDNV were engineered to express microRNAs that target host genes or to sequester host miRNA using antisense miRNA sponges (Liu *et al.*, 2016a). This strategy overcomes some of the challenges associated with expressing larger genes from these viruses and enables the use of RNAi, rather than effector molecules, for vector control.

MICROBES EXPRESSING RNAI - A promising alternative to paratransgenesis has emerged whereby microbes are engineered to deliver double stranded (dsRNA) to insects. RNAi is a powerful tool to manipulate transcription that has been used extensively to elucidate the function of many insect genes. In particular this technology has been extremely valuable in identifying mosquito pathways and genes that influence pathogen dynamics (Xi *et al.*, 2008; Garver *et al.*, 2009; Souza-Neto *et al.*, 2009) and other aspects of insect biology useful for mosquito control (Isoe *et al.*, 2011; Thailayil *et al.*, 2011; Figueira-Mansur *et al.*, 2013). The RNAi pathway is also a natural defense strategy used by insects to inhibit invading viral pathogens (Keene *et al.*, 2004; Sánchez-Vargas *et al.*, 2009), and therefore lends itself to development for applied pathogen control of arboviruses. This approach is very flexible in that potentially any gene in the vector could be manipulated. In addition, a vast array of interfering molecules can be delivered to the vector to manipulate gene expression, including short-hairpin RNAs (shRNA), long hairpin RNAs (lhRNA), artificial microRNAs (amiRNA) or miRNA sponges. Engineered

microbes could deliver multiple RNAi molecules, allowing several synergistic intervention strategies to be undertaken simultaneously, reducing the risk of evolution of resistance to a particular intervention approach. Theory predicts that viruses will not have the potential to evolve to such combinatorial intervention approaches (Leonard & Schaffer, 2005), and experimental evidence shows that polycistronic expression of multiple shRNA can effectively inhibit DENV (Xie *et al.*, 2013).

Delivery of RNAi to insects has been achieved with viruses, bacteria and yeast. For approaches targeting vector species, most strategies target host genes that when silenced induce mortality. These approaches can be considered as a novel species-specific insecticide. Other approaches have targeted genes that are important for reproduction, thereby reducing the fecundity of the insect. The use of bacteria for RNAi delivery is more complicated since the RNase III enzyme of the bacterium can degrade double stranded RNA (dsRNA). For many years, RNase III mutants of *Escherichia coli* have been used for RNAi silencing in the nematode *Caenorhabditis elegans* (Timmons *et al.*, 2001). Similar approaches with RNase III mutant *E. coli* are effective for RNAi delivery to *Ae. aegypti* mosquitoes and *R. prolixus* bugs (Taracena *et al.*, 2015; Whyard *et al.*, 2015), while a *R. rhodnii* RNase III mutant was used to express RNAi in *R. prolixus* (Whitten *et al.*, 2016). In contrast, wild type *R. rhodnii* bacteria were used to deliver RNAi molecules to Reduviid bugs that reduced fecundity of the insect (Taracena *et al.*, 2015). Similarly, fungi have been used to express RNAi targeting several essential host genes to kill agricultural pests (Chen *et al.*, 2015b; Murphy *et al.*, 2016). The use of bacterial or fungal microbes as RNAi delivery vehicles appear promising for vector control and the next challenges in this field will be to use this approach to interfere with pathogen development within a vector.

DEPLOYMENT STRATEGIES - Regardless of the nature of the anti-pathogenic phenotype, be it innate or engineered, a strategy to disseminate the symbiont effectively

through the vector population to have a meaningful effect on disease incidence is required. *Wolbachia*-based approaches have a clear advantage in this regard as the bacteria can manipulate host reproduction by CI to spread, often rapidly, through vector populations. For example, *Wolbachia* was established into *Ae. aegypti* populations in Cairns, Australia, by release of infected adults (Hoffmann *et al.*, 2011). Subsequent analysis of the infection frequency in mosquito populations two years after the release found the bacteria was near fixation at the release sites (Hoffmann *et al.*, 2014). Other strategies have been proposed for bacteria that do not manipulate host reproduction, and these may be self-perpetuating or require continual releases depending on the biology of the symbiont and host. As mentioned above, one elegant approach used in paratransgenic strategies of Triatomine bugs exploits the unique coprophagic probing tendencies of *R. prolixus* (Durvasula *et al.*, 1997). For readily culturable microbes, it has been suggested that dissemination of the microbe into mosquito populations could be achieved by spiking larval pools or by baiting sugar feeders (Schlein & Müller, 2015). For the former, the microbe would either need to be transstadially transmitted or the adult would need to imbibe the microbe soon after emergence from the pupal case. It appears that gut bacteria are cleared during metamorphosis between mosquito life stages (Moll *et al.*, 2001), but transstadial transmission may occur when other tissues like the malpighian tubules act as a reservoir for reinfection (Chavshin *et al.*, 2015). In semi-field cage experiments, both sugar feeding stations and release of infected males was shown to be an effective method to perpetuate *Asaia* through *Anopheles* generations (Mancini *et al.*, 2016). *Asaia* can be horizontally acquired and vertically transmitted, both maternally and paternally, which could perpetuate the infection (Favia *et al.*, 2007; Damiani *et al.*, 2008). A better understanding of the vertical and horizontal transmission of microbes and factors that influence microbiome homeostasis and composition is required before we can develop effective strategies for microbial release.

FUTURE DIRECTIONS FOR MICROBIAL CONTROL OF ARTHROPOD BORNE DISEASE

- Although there is a rich history of insect symbiosis research, many questions are yet to be resolved, particularly with regard to vector microbiomes. For successful utilization of microbes for applied control approaches several areas need to be addressed. Translating promising strategies that demonstrate that microbes can modulate vector competence in the lab to natural populations is a priority. For this to be achieved studies assessing the diversity of vector-associated microbes across diverse ecological niches is required. A related future direction is to examine both the host-microbe and host-microbe-pathogen tripartite interactions under differing environmental conditions such as temperature, as this variable has been shown to influence vector immunity and pathogen dynamics (Murdock *et al.*, 2012). While a particular control strategy may be successfully implemented under one set of environmental and ecological variables, this may not hold true where conditions differ.

Another important area of future research is in understanding the factors that influence how microbes are acquired, maintained, and transmitted by vectors. This knowledge is essential for developing effective methods to deploy symbionts into a population. Dissemination of a symbiont into a vector population may be hindered by microbial competition within the host. For example, *Wolbachia* and other bacterial microbes such as *Serratia* and *Asaia* are antagonistic to one other (Hughes *et al.*, 2014b; Rossi *et al.*, 2015; Zink *et al.*, 2015). Additionally, re-introduction of bacterial microbes into mosquitoes via a sugar meal was more successful when the native microbiota were suppressed by antibiotics, suggesting bacterial interactions in the gut dictate microbial colonization (Ramirez *et al.*, 2014). Cross kingdom interactions between bacteria and fungi, both positive and negative, were seen *Aedes triseriatus* and *Aedes japonicus* (Muturi *et al.*, 2016).

Microbial interactions have also be documented in tsetse flies and ticks (Boucias *et al.*, 2013; Wang *et al.*, 2013a; Fryxell & DeBruyn, 2016). As such, the issue of compatibility between microbial strategies could arise. For example, a *Wolbachia* based approach may interfere with an ISV strategy, as ISVs have recently been shown to be suppressed by *Wolbachia* antiviral activity (Schnettler *et al.*, 2016). Furthermore, paratransgenic approaches using *Asaia* or *Serratia* may not be compatible with *Wolbachia* applied approaches. While such an occurrence could be overcome by assessing the most suitable approach for a particular invention, strategies that perpetuate and drive through populations may expand geographically and therefore preclude the use of another technology elsewhere. Furthermore, the compatibility between microbial-based approaches and other contemporary and conventional vector control strategies should be investigated.

Another challenge with using microbes that possess native anti-pathogenic effects is determining the mechanism(s) by which they interfere with pathogens. Studies are providing insights into the mechanism(s) of pathogen interference of *Wolbachia* (Pan *et al.*, 2012; Caragata *et al.*, 2013; Zhang *et al.*, 2014), gut microbes (Azambuja *et al.*, 2004; Cirimotich *et al.*, 2011; Ramirez *et al.*, 2014), and fungi (Angleró-Rodríguez *et al.*, 2016; Valzano *et al.*, 2016), however a more comprehensive mechanistic understanding would facilitate attempts to forecast the long-term evolutionary response of the pathogen to the intervention and assist in determining the most effective deployment regime for a particular approach. While attempts have been made to predict these long-term interactions (Bull & Turelli, 2013), there are still unknown factors in these systems which makes these evaluations difficult. In contrast to this, the mechanism by which paratransgenic approaches inhibit pathogens is known as the effector molecule or RNAi cassette is engineered into the microbe. However, this means that all paratransgenic approaches have the unavoidable consequence that the microbe is genetically altered in some fashion.

For the ultimate utility of paratransgenic approaches, society needs to be receptive to this technology. Demonstrating the widespread benefits of these approaches by completing thorough and transparent research will enable societies and governments to make an informed decision of the risks and benefits of these novel control strategies. Further to this, the adoption of novel approaches to limit horizontal transfer of the transgene or the use of microencapsulation to contain microbes from environmental exposure will further enhance the safety of this technology (Arora *et al.*, 2015; Mandell *et al.*, 2015; Rovner *et al.*, 2015). The success of the *Wolbachia* strategy employed by the Eliminate Dengue Campaign can provide a blueprint for other microbial-based strategies to address ethical, social and logistical hurdles. In particular, this program has received wide-spread community acceptance that can be attributed to their comprehensive risk assessments and outstanding outreach and engagement efforts (McNaughton, 2012; McNaughton & Duong, 2014; Murray *et al.*, 2016).

SUMMARY - While conventional vector control strategies have reduced the burden of some VBD, novel strategies are required. Microbial-based strategies are gaining traction as an alternative means to control VBD, as microbes have several desirable properties for applied control strategies, particularly the ability to disseminate through vector populations. Coupling this with the propensity of symbiotic microbes to interfere with pathogen development in the host or by engineering microbes to modulate vector competence vectors, microbial strategies offer great promise for control of important VBDs.

Chapter 2 The microbiome of *Ae. aegypti* lines under different environmental laboratory conditions uniquely respond to distinct carbohydrates.

ABSTRACT

The microbiome alters many biological traits in mosquitoes, including vectorial capacity. As such, a greater appreciation of factors that influence microbiome acquisition and composition has the potential to be exploited for novel microbial-based control strategies. Like other insects, the microbiome of mosquitoes is variable and dynamic, and is affected by the environment, host and bacterial genetics, microbial interactions, and diet. While it is known that blood feeding alters the microbiome, little attention has been paid to how carbohydrate source directly influences microbiome composition; however, this is likely to be important as mosquitoes are known to feed on nectar. To explore how different sugars influence the microbiome of mosquitoes, we characterised the microbiome of two *Ae. aegypti* mosquitoes lines (Galveston and New Orleans) in two separate insectary environments reared on either sucrose, glucose, or fructose using 16S rRNA amplicon sequencing and qRT-PCR. Furthermore, we assessed ZIKV infection in the Galveston line to determine how potential alterations in the microbiome by sugar type affect vector competence. Regardless of diet, we found that mosquitoes from each location had distinct microbiomes and the New Orleans line had a higher bacterial load compare to the Galveston line. Interestingly, the carbohydrate source only changed the commuinty structure of the microbiome in the New Orlean line but not the Galveston line, although specific bacterial taxa were signicantly altered in response to sugar treatment in both lines. Vector competence assays conducted in parrellel with microbiome characterization experiment in the Galveston line found no significant differneces in vector competence to ZIKV, which was an intutive finding given that mimimal changes were seen in the

microbiome in response to differing carbohydrate sources. Our findings demonstrate the importance of host diet on the microbiota and show that alterations in the microbiome occur in a line specific manner, likely due to the ability of particular microbiota, which were unique to the New Orleans line, to utilized different carbohdrate sources. Our results highlight that the microbiome of certain mosquitoes lines are susceptible to preturbation by sugars and that the composition of carbohydrates in nectar could contribute to microbiome variabilty in mosquito vectors.

INTRODUCTION.

The bacterial microbiome of mosquitoes is complex, dynamic, and variable across species and populations (Wang *et al.*, 2011, 2017; Zouache *et al.*, 2011; Osei-Poku *et al.*, 2012; Buck *et al.*, 2016; Coon *et al.*, 2016; Pennington *et al.*, 2016; Hegde *et al.*, 2018). Like many other insect hosts, environment, diet, host and bacterial genetics, and microbial interactions all influence the mutable microbiome (Dickson *et al.*, 2017b; Hegde *et al.*, 2018; Minard *et al.*, 2018; Muturi *et al.*, 2018). As most mosquitoes are hematophagous, they require a blood meal for egg development. The rapid influx of nutrients from the blood meal dramatically alters the microbiome with the density of bacteria increasing but species diversity decreasing (Kumar *et al.*, 2010; Oliveira *et al.*, 2011; Wang *et al.*, 2011; Terenius *et al.*, 2012). While the response of the microbiome to blood feeding has been documented in several mosquito species (Wang *et al.*, 2011; Tchioffo *et al.*, 2016), little attention has been paid to other nutritional sources, such as sugars and nectars that could potentially impact the composition and abundance of the microbiome.

Diet influences both the diversity and composition of microbiota in a range of animals. In both juvenile rats and humans, altering sugar consumption or type led to changes in specific bacterial taxa. In insects, shifting flies from a molasses-based to a starch-based diet resulted in significant alterations in the microbiome (Sharon *et al.*, 2010). Supplementing termites with sugars and amino acids caused a general reduction of bacterial species richness while secondary metabolites significantly altered diversity by increasing the levels of *Firmicutes* and *Spirochetes* (Huang *et al.*, 2016). Bacteria in honeybees fed on single carbon sources, specifically fructose, glucose, or sucrose exhibitied differences in their ability to metabolize these sugars (Lee, 2018). Additionally, indirect evidence that diet influences the microbiome of mosquitoes can be inferred from colonizing field lines in the laboratory, although other environmental factors are also at

play in these scenarios. Chandler and coworkers (2011) demonstrated that the microbiome of three distinct field-collected fly lines, which had divergent diets in the wild, converged when reared on the same diet in the lab. Similarly, genetically diverse *Aedes aegypti* lines reared in a common insectary environment had comparable microbiomes (Dickson *et al.*, 2017a). Taken together, these results suggest that diet, and particularly carbon source, have the potential to alter the composition of the microbiome, however, despite this, little is known about the role of carbohydrates on the mosquito microbiome. This is a critical question in vector biology as mosquitoes use a range of plant nectars as a food source.

Plant nectars contain a mixture of carbohydrates, mainly, fructose, glucose, and sucrose (Wykes, 1952; Chalcoff *et al.*, 2006). Sucrose often makes up the highest proportion of sugar (Chalcoff *et al.*, 2006; Lohaus & Schwerdtfeger, 2014; Rodríguez-Riaño *et al.*, 2014). For many mosquito species, nectar feeding was presumed to be the main source of nutrients for both males and females (Magnarelli, 1978; Foster, 1995). However, it was alternatively hypothesized that female *Ae. aegypti* acquired nutrients solely from blood meals (Edman *et al.*, 1992, 1997; Harrington *et al.*, 2014), although there is evidence that females also exploit nectar sources (Martinez-Ibarra *et al.*, 1997; Chen & Kearney, 2015; Qualls *et al.*, 2016). When adults were reared on plants in a laboratory setting, *Anopheles*, *Aedes,* and *Culex* mosquitoes had increased longevity and increased egg production compared to their sucrose fed counterparts (Mostowy & Foster, 2004; Manda *et al.*, 2007; Gu *et al.*, 2011; Chen & Kearney, 2015). Given that microbiota can also influence these phenotypes, it is tempting to speculate these effects could be mediated by the microbiome if nectar feeding altered certain bacterial taxa.

As many mosquito species are known nectar feeders, the standard practice across many insectaries is to maintain laboratory colonies on a single sugar source, often sucrose (Johnson, 1947; Kuno, 2010; Imam *et al.*, 2014). Occasionally, glucose has been used as

the carbohydrate source for rearing mosquitoes (Gonçalves *et al.*, 2014). Although sucrose is the predominant component of nectar, maintaining lab mosquitoes on a single sugar source poorly replicates their diet when nectar feeding in the field as it fails to account for the impact the other sugars may be contributing to the microbiome composition and diversity. Mosquitoes reared under these conditions are then utilized in experiments attempting to understand aspects of mosquito biology such as vector competence. This practice brings into question the biological relevance of these studies and further illustrates differences between laboratory-reared and mosquitoes out in the field.

To address this gap in knowledge, we reared *Ae. aegypti* mosquitoes on either fructose, glucose, or sucrose for seven days and characterized their microbiome. In parallel, we explored the effect of sugar on vector competence to Zika virus (ZIKV). Here we show that the microbiomes of mosquitoes from two locations are divergent and these microbiomes response differently to carbohydrate sources. Thus, we demonstrate that altering the mosquito carbohydrate source is a simple way to perturb their microbiome. These results have implications for understanding factors that promote the variability of the microbiome of mosquito populations and provide potential avenues to modulate the microbiome of mosquitoes for novel microbiota-based vector control strategies.

METHODS

Mosquito rearing

Two to three day-old female *Ae. aegypti* mosquitoes (Galveston, third generation reared in the UTMB insectary (F3) and New Orleans line (NO), reared for over 13 years in the LSTM insectary) were fed either 10% glucose, fructose, or sucrose (Sigma-Aldrich). After 7 days, ~20 mosquitoes from each group were collected and DNA extracted. To extract DNA, mosquitoes were surface sterilized (5 min in 70% ethanol followed by 3 washes in 1X PBS each for 5 min) and total DNA was extracted using the NucleoSpin Tissue kit (Clone Tech).

Quantification of bacterial density

Total bacterial load in mosquitoes was assessed by qPCR using genomic DNA (gDNA) as a template. Relative abundance was assessed by amplifying the bacterial 16S rRNA gene using universal bacterial primers (Kumar *et al.*, 2010) and S7 a single copy mosquito gene (Isoe *et al.*, 2011). The relative abundance of 16S rRNA gene copy number to the endogenous mosquito control was analyzed to determine the total bacterial load in these samples. PCR cycling conditions and primers were described by Hegde and coworkers (2018).

Microbiome sequencing and bioinformatics analysis

High-throughput sequencing of the bacterial 16S rRNA gene was performed using gDNA isolated from each sample. From the Galveston line, we analyzed 21 fructose fed mosquitoes, 20 glucose and 23 sucrose, while from New Orleans we analyzed 24 fructose fed mosquitoes, 25 glucose and 23 sucrose fed mosquitoes. Sequencing libraries for each isolate were generated using universal 16S rRNA V3-V4 region primers (Klindworth *et*

al., 2013) in accordance with Illumina 16S rRNA metagenomic sequencing library protocols. The samples were barcoded for multiplexing using Nextera XT Index Kit v2. Sequencing was performed on an Illumina MiSeq instrument using a MiSeq Reagent Kit v2 (500-cycles).

To identify the presence of known bacteria, sequences were analyzed using the CLC Genomics Workbench 12.0.1 Microbial Genomics Module (http://www.clcbio.com). Reads containing nucleotides below the quality threshold of 0.05 (using the modified Richard Mott algorithm) and reads with two or more unknown nucleotides or sequencing adapters were trimmed out. All reads were automatically trimmed for adapter and quality filtration for subsequent operational taxonomic unit (OTU) classification. Reference based OTU picking was performed using the SILVA SSU v123 99% database (Quast *et al.*, 2012). Sequences present in more than one copy but not clustered to the database were then placed into de novo OTUs (97% similarity) and aligned against the reference database with 80% similarity threshold. Chimeras were removed from the results if their absolute crossover cost was 3 using a k-mer size of 6. Alpha diversity was measured using Shannon entropy, rarefication sampling without replacement, and with 100,000 replicates at each point. Beta diversity was calculated using the Bray-Curtis diversity measure.

Zika virus infections

Two week old female *Ae. aegypti* mosquitoes that have been reared on either 10% sucrose, fructose, or glucose were orally infected with ZIKV (Mex 1-7 isolate) at 6.5 x 10^6 focus forming units (FFU)/ml) in a sheep blood meal (Colorado Serum Company) (Saldaña *et al.*, 2017). Following the blood meal, mosquitoes were then reared on their respective sugar source. At 10 days post infection (dpi) mosquitoes were collected and processed for viral quantification. Carcasses and legs, which were processes separately, were

homogenized in 500µl tissue culture media, and then stored at -80°C prior to viral titer assessment. Virus density was measured by focus forming assay (FFA) performed on infected Vero cells (seeded at $5x10^4$) with 10^{-1}, 10^{-2} and 10^{-3} serial dilutions, after a three day incubation period (Vilcarromero *et al.*, 2014; Roundy *et al.*, 2017a).

RESULTS AND DISCUSSION

Host diet plays a pivotal role in shaping the microbiome composition and abundance in many organisms including insects (Crotti *et al.*, 2010; Hibbing *et al.*, 2010; Engel & Moran, 2013; Townsend *et al.*, 2019). To explore the impact of diet in *Ae. aegypti* mosquitoes, and specifically address how carbohydrate source influences the microbiome, we reared *Ae. aegypti* on three distinct sugar sources and characterized their microbiomes using culture independent approaches. We sequenced amplicons of the V3/V4 region of the 16S rRNA gene from 135 individuals, of which 64 were the Galveston line reared in the UTMB insectary and 71 the New Orleans line reared in the LSTM insectary. After quality filtering 4,462,046 reads were assigned to operational taxonomic units (OTUs) and on average there were 38,466 reads per mosquito sample. Rarefaction curve analysis indicated that the sequencing depth was sufficient to identify all OTUs in each individual (Figure 1). The Galveston line was dominated by bacteria in the *Enterobacteriaceae* family including *Citrobacter*, *Raoultella*, and to a lesser extent *Serratia*, while the New Orleans line was predominately infected by *Pseudomonas*, *Asaia,* and an *Acinetobacter* (Figure 2). The former two taxa were also found in the Galveston line but were not dominant species. As 16S rRNA amplicon-based studies are prone to contamination, we ran both negative extraction and positive controls. Analysis of these control samples indicated there was minimal contamination in our samples.

Sugar Type Alters the Microbial Diversity in Mosquitoes

To determine how sugar type altered the species richness in mosquitoes, we examined the alpha diversity using the Shannon's diversity index metric (Shannon, 1948). There were no significant differences when comparing sugar type within a location or between lines for a particular sugar (Figure 3A), suggesting that sugar source had minimal impact on microbiome species richness. To explore differences in the microbiome

community structure, we measure the beta diversity between each condition using Bray-Curtis non-metric Multidimensional Scaling (nMDS), which takes into account the absence/presence and relative abundance diversity of OTUs in each sample but does not consider phylogeny of the bacteria. It was evident that clustering was dictated by mosquito line as treatments within the Galveston and the New Orleans lines grouped together (Figure 3B; PERMANOVA; line $P < 0.0009$); however, sugar type and the interaction between sugar and line were also significant (PERMANOVA; sugar $P < 0.0009$; interaction $P < 0.0009$). When each line was examined independently, sugar type was not significant in the Galveston line (PERMANOVA; $P = 0.051$) (Figure 3C) but was significant in the New Orleans line (PERMANOVA; $P < 0.0009$) (Figure 3D), suggesting the microbiome of the New Orleans line had the capacity to respond to sugar sources while the microbiome of the Galveston line did not. It is also possible that genetic differences in the host mediated these effects. Given we saw a response in the New Orleans line, we then performed pairwise PERMANOVA on the Brays Curtis distance matrix to determine differences in the microbiomes due to sugar type. Mosquitoes reared on glucose has a significantly different microbiome compared to both the sucrose ($P < 0.003$) and fructose ($P < 0.0075$), while there was no difference in the microbiome between sucrose and fructose fed mosquitoes ($P = 0.5$) (Figure 3D).

It is evident that environment alters the mosquito microbiome (Rani *et al.*, 2009; Dickson *et al.*, 2017b; Mwadondo *et al.*, 2017; Hegde *et al.*, 2018). By comparing microbiome datasets of lab-reared mosquitoes it is apparent that mosquitoes reared in different insectaries have divergent microbiomes (Linenberg *et al.*, 2016; Romoli & Gendrin, 2018), but to our knowledge a direct comparison controlling for variables known to influence microbiome sequencing addressing the influence of the insectary environment has not been undertaken. Here we show that the microbiomes of the New Orleans and Galveston lines are profoundly different, likely due to environmental factors found at each

insectary. Importantly our samples were sequenced in the same manner, meaning this comparison is more appropriate than comparing various studies that have used different sequencing approaches (Pollock *et al.*, 2018). Of course, these lines potentially have host genetic differences that could affect microbiota composition, and a more thorough investigation would rear each line in the reciprocal insectary; however, this was not the focus of our study. These data here, taken together with the findings that rearing diverse mosquitoes into a common insectary converges the microbiome (Coon *et al.*, 2016; Dickson *et al.*, 2017a), provides compelling evidence that the laboratory environment profoundly alters the microbiome of mosquitoes.

To identify which bacteria are likely contributing to differences in microbiome community structure of each mosquito sugar treatment, we completed a pairwise Analysis of Composition of Microbiomes (ANCOM) (Mandal *et al.*, 2015) to identify bacterial genera significantly different in abundance between treatments. Corroborating our nMDS results, we see a greater number of bacterial taxa significantly ($P < 0.05$) altered by sugar treatment in the New Orleans line compared to the Galveston (Table 1). *Pantoea* was elevated, while *Sphingobacterium* was suppressed in the glucose treatment compared to the two other sugars in the New Orleans line. Sucrose also reduced *Asaia* density when compared to both fructose and glucose. In the Galveston line, *Aeromonas* was elevated in the sucrose treatment compared to the other sugar treatments while *Serratia* was significantly altered across all treatments, with highest titers seen in the fructose, then glucose, and the lowest density in the sucrose treatment. A similar finding was reported by Solé and co-workers (2000), whereby they measured the acidification of media in response to different sugar types, demonstrating that *Serratia* could preferential metabolize sugars, although we saw a preference for fructose over glucose. Both our current study and that of Solé and co-workers (2000), agree that sucrose is a less preferred sugar source for *Serratia*, which potentially explains our previous results whereby another *Ae. aegypti* Galveston line,

which had been reared solely on sucrose for many years, appeared to be void of *Serratia* (Hegde *et al.*, 2018).

While little is known regarding the metabolism of gut-associated bacteria of mosquitoes, insights can be inferred from their free-living relatives. *Asaia* which has been characterized as a common and stable microbe in mosquitoes (Favia *et al.*, 2007; Crotti *et al.*, 2010; Damiani *et al.*, 2010; Hegde *et al.*, 2018), belongs to the group of bacteria known as Acetic Acid Bacteria (AAB). AAB, commonly found in sugar rich environment including food, beverages, and plants (Crotti *et al.*, 2010; Chouaia *et al.*, 2014), have been shown to preferentially metabolize glucose as their carbon source but are also known to metabolize other sugars including fructose (Mamlouk & Gullo, 2013). This is in support our findings, in which *Asaia* had a greater abundance in the glucose treatment and a minimal change in the fructose treatment when compared to sucrose. As previously noted, *Comamonas* and *Sphingobacterium* both responded favorably to sucrose. With regard to *Comamonas*, the significant difference observed between glucose and sucrose could likely be attributed to the lack enzymes necessary for glucose metabolism (Wu *et al.*, 2015). On the other hand, *Sphingobacterium* demonstrates an affinity to both fructose and sucrose as opposed to glucose. However, this is contrary to reports in the literature which describe many species of *Sphingobacterium* primarily utilizing glucose as a carbon source (Kim, 2006; Ten *et al.*, 2006; Huys *et al.*, 2012; Li *et al.*, 2016b). Additional conflicting reports have both glucose and sucrose (Ahmed *et al.*, 2014), or fructose (Ten *et al.*, 2006) listed as not being fermented by *Sphingobacterium*. These discrepancies brought up by the literature are likely due, in part, that many of the species characterized are free-living environmental organisms, which had acquired traits for survival in specialized environments, rather than co-evolving with a host. This idea is supported by microbiome analyses in bees and weevils that have shown specific bacterial taxa have specialize niche roles, in which these

microbiota are able to preferentially metabolize different carbohydrates (Engel *et al.*, 2012, 2014; Moran, 2015; Zheng *et al.*, 2016; Muhammad *et al.*, 2017).

Sugar Type has a Line Specific Influence on Bacterial Load

In addition to the impact that sugar type played on microbiome composition, we also explored the effect each sugar had on the total bacterial load in these mosquitoes. Although high throughput sequencing does offer some level of characterization of the species composition in the microbiome, it only provides a relative measure of bacterial density between samples. Therefore, to obtain an estimate of the total bacterial load in each *Ae. aegypti* by sugar treatment group, we completed qPCR on mosquitoes with universal eubacterial primers that broadly amplify bacterial species (Kumar *et al.*, 2010). Similar to the beta diversity results, large variation was seen in the New Orleans line compared to the Galveston line. When mosquitoes were grouped by location, we observed New Orleans had significantly higher total bacterial loads compared to Galveston (Mann-Whitney $p=0.007$). (Figure 4A). No significant difference was seen between sugar types within a location (Kruskal-Wallis test $p>0.05$) (Figure 4B). However, when we compared the bacterial loads of mosquitoes from each line by sugar treatment, we observed that the mosquitoes from New Orleans had significantly more bacteria than those from Galveston, when fed on either glucose (Mann-Whitney test $p=0.042$) or sucrose (Mann-Whitney test $P=0.0009$), while no difference was observed for fructose fed mosquitoes (Figure 4C).

These results suggest there is likely a relationship between microbial taxa and load in each of the mosquito lines and that bacteria which are present in New Orleans line, but absent in the Galveston line, may be better suited for metabolizing either glucose or sucrose resulting in a higher bacterial density in these mosquitoes (Lee, 2018). Further work exploring the ability for mosquito microbiota to metabolize specific carbohydrates would

be needed to confirm this hypothesis. In addition to location, other differences between the two mosquito lines used for this study include their time of colonization and passage history. The Galveston line was recently established by collecting host-seeking females. This lineage has only been reared for 3 generations, spending less than a years' time under standard laboratory conditions. On the other hand, the New Orleans mosquitoes are a highly colonized line, which originates from the CDC New Orleans lineage and have been reared in the Liverpool School of Tropical Medicine insectary for more than 13 years.

Zika virus infection

Microbiota that reside within the mosquito gut have the capacity to alter vector competence. Both composition and total bacterial load have previously been shown to play a role in vector competence in other studies (Kent *et al.*, 1996; Zouache *et al.*, 2011; Boissière *et al.*, 2012; Lu *et al.*, 2012; Ramirez *et al.*, 2012). The observation of a highly variable microbiome within mosquito populations raise the question of identifying which microbes are specifically involved in vector competence (Gonçalves *et al.*, 2014). Therefore, to explore how perturbing the microbiome affects vector competence in *Ae. aegypti,* we offered an infectious ZIKV blood meal to mosquitoes maintained on either fructose, glucose, or sucrose. The vector competence assay was done in Galveston line in parallel with our microbiome characterization. We observed no significant differences in viral titers across the different sugar treated cohorts (Kruskal-Wallis; $p>0.05$) (Figure 6). The findings are in line microbiome data as we would not expect to see profound changes in ZIKV infection as there was minimal alteration in the microbiome community structure in response to sugar.

Our data suggest that bacteria in the Galveston line that were modulated by sugar have little impact on ZIKV, although these effects could be dissipated by other bacteria or

interactions between bacteria. *Serratia,* which is a member of *Enterobacteriaceae,* has been shown in studies to enhance arboviral infections including dengue virus (DENV) in *Ae. aegypti* mosquitoes (Apte-Deshpande *et al.*, 2012, 2014; Wu *et al.*, 2018). Further to this, my work (described in chapter 3) has demonstrated that bacteria belonging to the family *Enterobacteriaceae* play a role in facilitating ZIKV infections in mosquitoes. While the relative abundance of *Serratia* was altered by sugar type, overall the proportion of *Serratia* in the microbiome in the Galveston line was relatively low (ranging from 0.006% – 10% relative abundance) yet were at a much higher density in the New Orleans line (ranging from 26% – 48% relative abundance). Regardless of these observed increases, *Serratia* appeared to have minimal effect on ZIKV infection at these densities. A comparison of virus infection between the Galveston and New Orleans lines would help to determine the role of *Serratia* and other bacteria in ZIKV infections, while vector competence assays in mono-axenic lines would aid in dissecting the influence of bacterial taxa on vector competence. Experiments could also be undertaken to determine if there is a specific density threshold of *Serratia* (or other bacteria) that impacts on ZIKV infection. Additionally, further examination of the specific species or strains of *Serratia* may also explain our findings, given that there are nearly twenty different species and to this point only two, *S. odorifera* and *S. marcescens*, have been implicated in enhanced arbovirus susceptibility in *Ae. aegypti* (Apte-Deshpande *et al.*, 2012, 2014; Wu *et al.*, 2018).

Our data also suggest that sugar has minimal effect on host responses to ZIKV infection. However, given that the microbiome of the New Orleans line was more receptive to sugar type, it would be intriguing to challenge these mosquitoes with ZIKV when reared on each of the three sugars. While we contemplated conducting vector competence experiment studies on the New Orleans line at UTMB, this was discounted, as this line would have likely acquired the Galveston microbiome when reared in the UTMB insectaries. As such, it is more appropriate to conduct these additional vector competence

studies *in situ* at LSTM. These experiments are will be undertaken in the future but are beyond the scope of our current study.

Summary

In conclusion, we show that *Ae. aegypti* lines reared in different insectaries possess distinct microbiomes and that sugar type affected the community structure of the microbiome of these mosquitoes in a line specific manner. When microbiome alterations occurred, mosquitoes reared on either sucrose or glucose had the most divergent microbiome. Altering the sugar source used to rear mosquitoes offers a simple approach to perturb the microbiome. These findings have implications for understanding the considerable variation observed in the mosquito microbiome, which potentially explaining variability in transmission of pathogens and further highlight difference between field and colonized mosquitoes which rear mosquitoes on single sugar source.

Table 1. ANCOM Analysis of Differentially Abundant Bacteria in Response to Sugars.

This table outlines the fold change of significantly different responses by bacterial taxa to sugar type within each location $p<0.05$.

		Fructose v Sucrose	Glucose v Sucrose	Fructose v Glucose
Strain	**Genus**	**Fold Change**	**Fold Change**	**Fold Change**
Galveston	*Aeromonas*	-2.099144305	-3.254407301	
	Chryseobacterium	8.207393149	8.520092462	
	Serratia	5.864460942	4.357872051	1.506588891
New Orleans	*Asaia*	3.104982564	3.659856371	
	Comamonas		-2.61372513	
	Flavobacterium	5.881101427		
	Pantoea		4.029587067	-3.295404039
	Sphingobacterium		-2.628157238	4.885469704

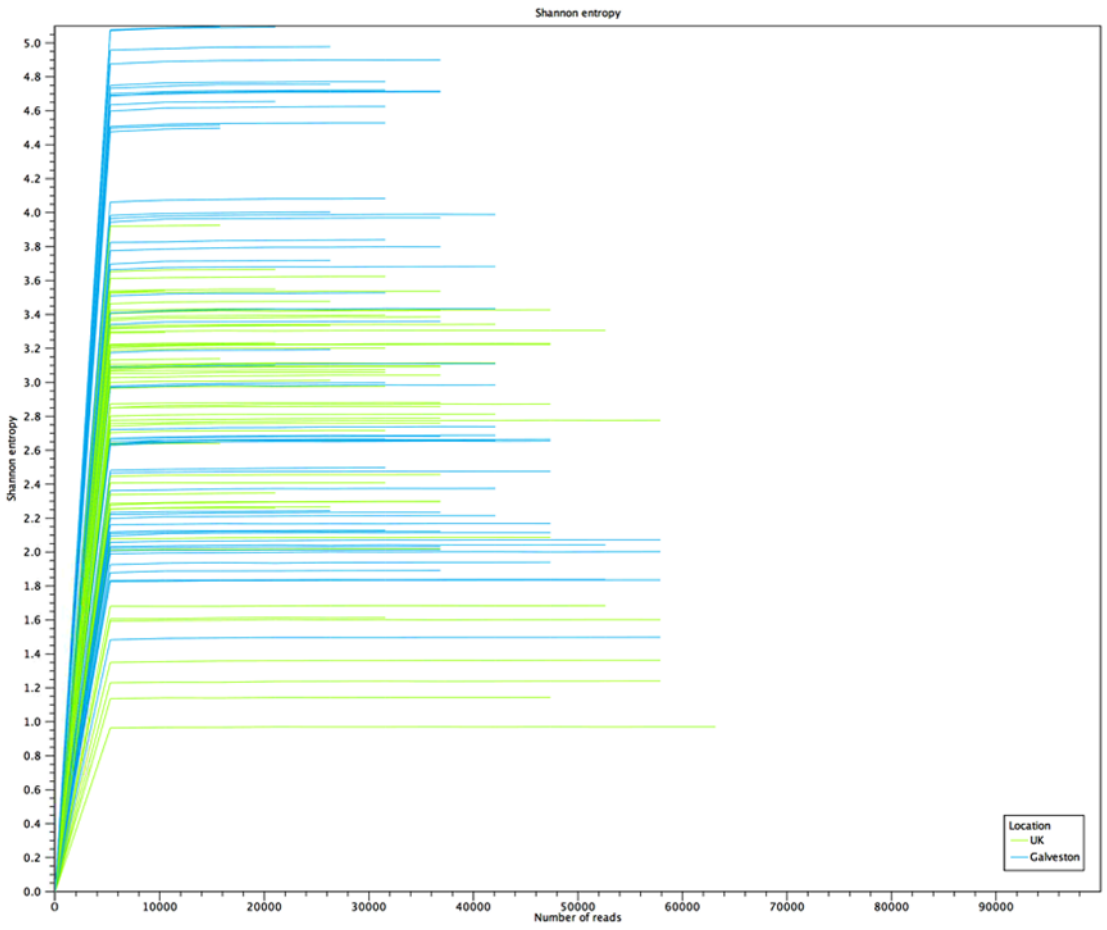

Figure 1. Rarefaction curve analysis.

Reads per mosquito sample were assigned to operational taxonomic units (OTUs). Rarefaction curve analysis indicated that the sequencing depth was sufficient to identify all OTUs in each individual sample.

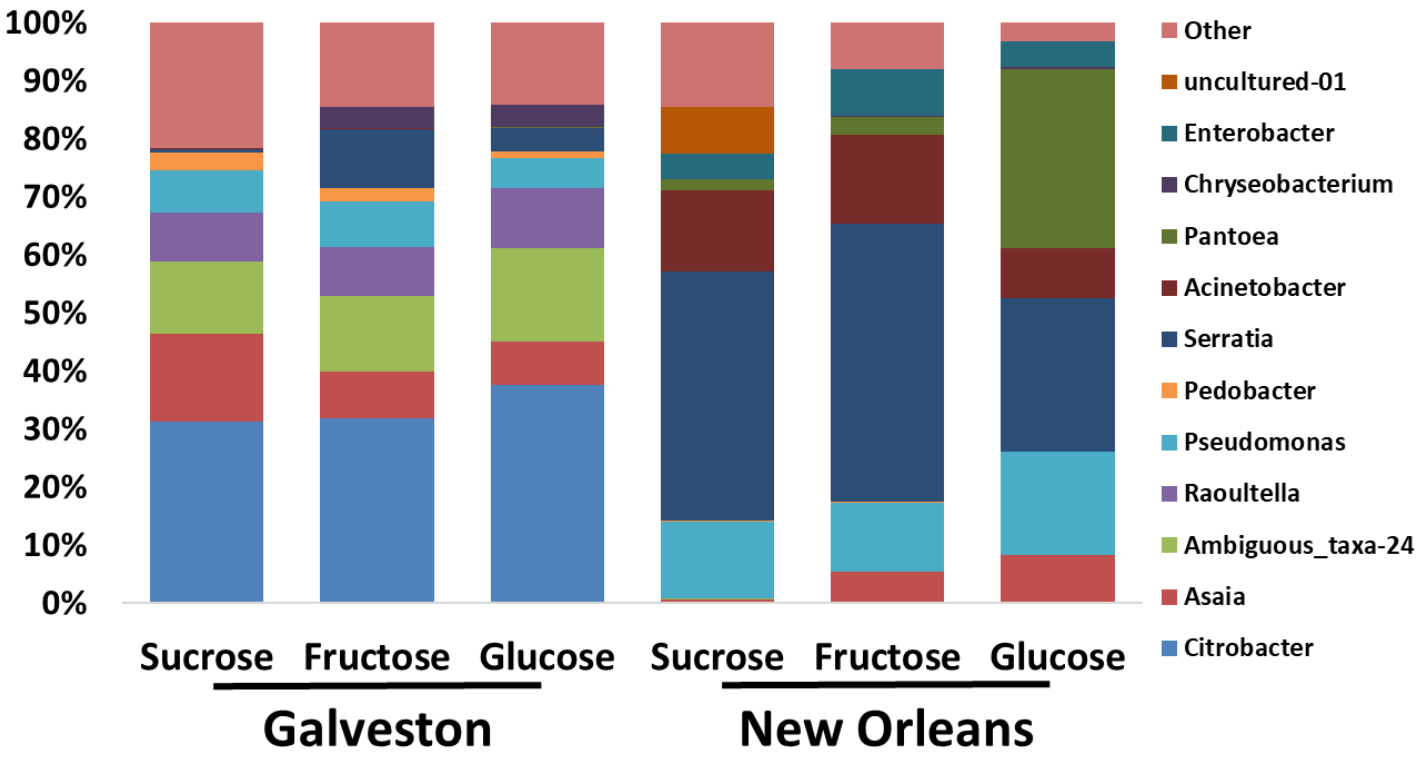

Figure 2. Relative abundance of microbiota in mosquitoes fed on different sugar types by location.

Relative abundance of bacterial genera present at 0.1% and above for each sugar type by location.

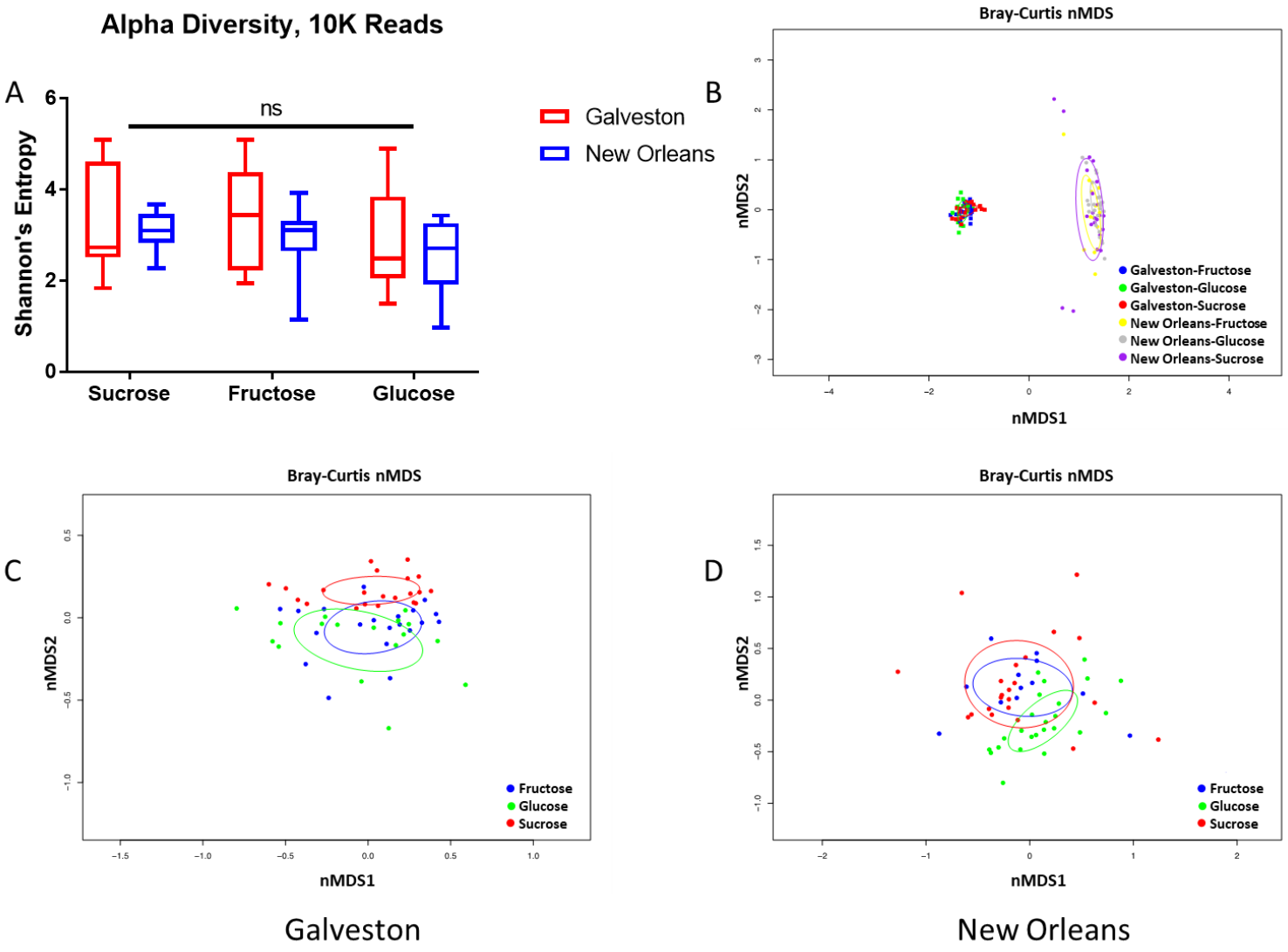

Figure 3. Microbiome diversity in sugar fed mosquitoes by location.

(A) Shannon diversity indices at the genus level for all mosquitoes by sugar type and location. A Tukey's multiple comparison test was used to determine significance between sugar types for Galveston and New Orleans mosquito populations. (B-D) Non-metric Multidimensional Scaling (genus level) using Bray-Curtis dissimilarity, comparing identified OTUs within a group. PERMANOVA significance values for pairwise comparison B (mosquito line $P < 0.0009$), (sugar type $P < 0.0009$) (sugar and line $P < 0.0009$) C (sugar in Galveston $P = 0.051$) D (sugar in UK line $P < 0.0009$).

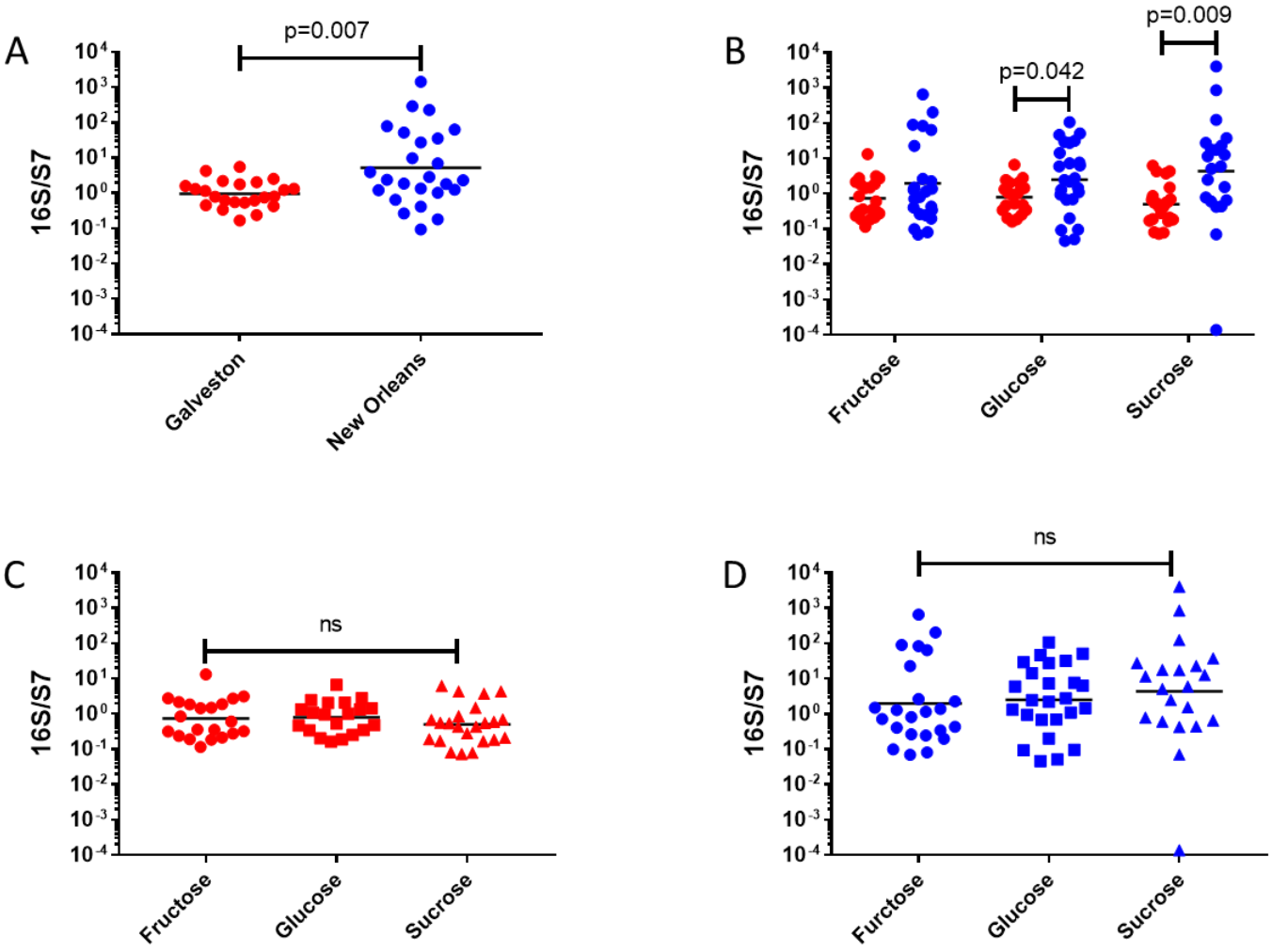

Figure 4. Total bacterial load in mosquitoes.

Comparison of bacterial load for each sugar type by location Galveston (red) or New Orleans (blue). Bacterial load is represented as a ratio between 16S rRNA gene copies to S7 copies (*Ae. aegypti*) gene. All graphs represent different comparisons of the same data set. (A) Pooled samples by location. (B) Comparison of sugar type between locations. (C-D) Comparison of sugar type within a location.

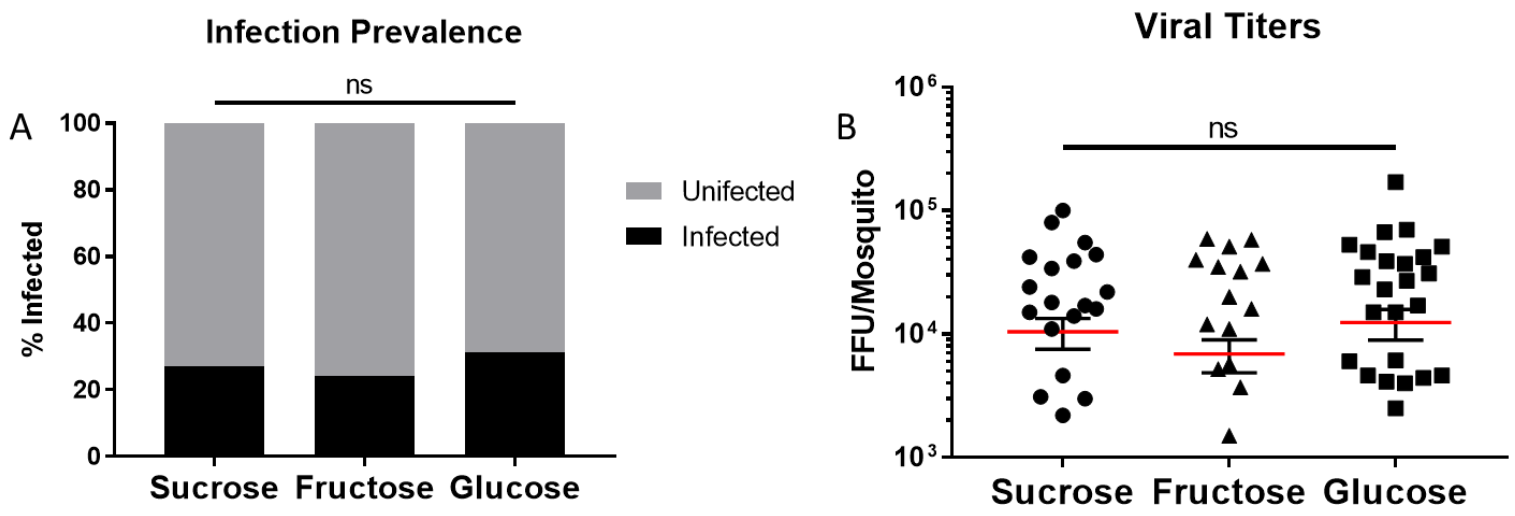

Figure 5. ZIKV infection in Galveston mosquitoes fed on different sugars.

Viral infection and titers were quantified by focus forming assay. (A)The infection prevalence was assessed by Chi squared test. (B) Kruskal-Wallis test with a Dunn's multiple comparison test determined no significant difference between treatments.

Chapter 3 Complex reciprocal interactions occur between the microbiota and Zika virus within lab-reared and field-collected *Aedes aegypti* mosquitoes.

ABSTRACT

Aedes aegypti mosquitoes are the primary vectors of several medically important viruses including Zika virus (ZIKV) and dengue virus (DENV). Recent outbreaks of these and other arboviruses illustrate a growing need for novel approaches to vector control. The microbiome of mosquitoes is known to influence susceptibility to infection of viral pathogens, as a result of the intimate relationship with both the host and the pathogens they transmit. Characterization of this tripartite interaction between the host, microbiota and pathogen will allow us to better understand how individuals in a population might be more susceptible to infection while others are refractory. In this study, we aimed to determine the extent to which the resident microbiome of *Ae. aegypti* mosquitoes influences infection and transmission of ZIKV by this vector. To address this question, we perturbed the microbiome of lab-reared *Ae. aegypti* with oral antibiotic treatments or by supplementing specific bacteria prior to infection with ZIKV. Intriguingly, we found mosquitoes administered with antibiotics had lower ZIKV titers compared to their untreated counterparts, regardless if the virus was offered in a blood meal or intrathoracically microinjected, indicating this effect may be mediated by the host. Antibiotic treatment also resulted in significant reduction in ZIKV titers for infected mosquitoes, regardless of decreased activation of immune gene regulators of the JAK/STAT (*STAT1*) and IMD (*Rel2*) innate immune pathways. 16S microbiome sequencing revealed that antibiotic treatment in mosquitoes reduced bacteria from the *Acetobacteraceae* and *Enterobacteriaceae* families, suggesting that these bacteria may be facilitating ZIKV infection. After confirming microbiota alter ZIKV infection, we examined how mosquito

microbiome variability between individuals within a population may alter virus infection dynamics. Here we collected host seeking *Ae. aegypti* mosquitoes from different field sites in Texas (Austin, Brownsville and Galveston). Mosquitoes were immediately offered an artificial ZIKV infectious blood meal and after 10 days post feeding mosqutioes were assayed for infection status as well as microbiome composition. Austin ZIKV-infected mosquitoes had a different microbiome from uninfected mosquitoes; however, no differencess were observed for mosquitoes from the other two sites. Microbiome comparisons of ZIKV infected mosquitoes from Austin revealed higher levels of *Enterobacteriaceae* when compared to unifected mosquitoes, corroborating our lab-reared mosquito findings. In addition, we noted ZIKV exposure resulted in altered mosquito microbiomes when compared to the blood fed control group of mosquitoes. Moreover, this alteration was observed in exposed mosquitoes regardless of their infection status. We disscuss these findings in the context of how the microbiome could cause variation in vector competence, in addition to the potential for developing novel microbial control strategies to reduce mosquito-borne disease.

INTRODUCTION

Mosquitoes are vectors of medically important human pathogens, including recurrent outbreaks of arboviruses like Zika virus (ZIKV), Yellow Fever virus (YFV) and chikungunya virus (CHIKV). Outbreaks caused by these arboviruses highlight the fact that traditional vector control strategies currently employed around the world are waning in their efficacy. The microbiome of mosquitoes has been shown to play a significant role in shaping many aspects of their natural biology, which has led to enthusiasm for studying ways to exploit microbiota for vector control.

Given that microbes can profoundly affect arboviruses, it is tempting to speculate that contrasting vector competence results could be due to differences in the microbiome. Indeed recently it was reported that the same strains of mice acquired from different vendors differed in their susceptibility to pathogens due to their distinct microbiomes (Velazquez *et al.*, 2019). However, while most efforts in mosquitoes have concentrated on identifying microbes that induce refractory phenotypes against pathogens, it is important to consider that complex tripartite interactions dictate both virus infection, microbiome composition and abundance outcomes. (Ramirez *et al.*, 2012; Hegde *et al.*, 2015). Therefore, when correlating the microbiome to pathogen infections, it is critical to understand the complex interplay between these microbes. For example, CHIKV infection has been shown to modulate the microbiome in *Aedes (Ae.) albopictus* mosquitoes by stimulating an increase in *Enterobacteriaceae* while decreasing *Rhodobacteraceae, Bradyrhizobiaceae* and *Wolbachia* (Zouache *et al.*, 2012). Likewise, *Culex (Cx.) pipiens* mosquitoes either exposed to or infected with West Nile virus (WNV) resulted in a significant increase in *Serratia sp.* bacteria and, similar to CHIKV, suppressed the abundance of *Wolbachia* in the mosquito gut (Zink *et al.*, 2015). In one study, ZIKV infection of *Ae. aegypti* mosquitoes stimulated an increase of *Rhodobacteraceae* and

Desulfuromonadaceae bacterial families (Villegas *et al.*, 2018). Previous work has documented up-regulated immune pathways in response to gut microbiota, leading to reduced viral infection in some mosquitoes (Ramirez *et al.*, 2012; Barletta *et al.*, 2017). These reports showed different bacteria are able to induce mosquito refractoriness to DENV by either influencing the immune system (Ramirez *et al.*, 2012) or through the secretion of anti-pathogenic molecules (Ramirez *et al.*, 2014). Furthermore, the bacteria *Serratia marcescens* has been described as being able to secrete proteins that increase mosquito permissiveness to DENV (Wu *et al.*, 2018). These findings, in addition to other influential factors including host genetics, lab environment and methods used to study microbial impacts, help explain the mixed results across the vast numbers of mosquito vector competence studies (Gubler & Rosen, 1976; Tesh *et al.*, 1976; Tabachnick *et al.*, 1985; Bennett *et al.*, 2002; Ebel *et al.*, 2005; Kilpatrick *et al.*, 2010; Cox *et al.*, 2011; Gonçalves *et al.*, 2014; Roundy *et al.*, 2017b), although other factors, including host genetics, lab environment and methods may also contribute to this variation. Another factor to consider is the strain of virus being used in these studies. A number of reports have highlighted how different strains and linages of viruses often demonstrate variability in infection rates and vector competence (Weger-Lucarelli *et al.*, 2016; Ciota *et al.*, 2017; Pompon *et al.*, 2017; Roundy *et al.*, 2017a; Veronesi *et al.*, 2018).

One way to study the mosquito microbiome is to decrease or eliminate bacteria through the oral administration of antibiotics in sugar meals, and to characterize the effects of the absent or reduced bacterial groups. For example, antibiotics were used to deplete microbiota in *Wolbachia* studies to illustrate the role of the microbiome in preventing vertical transmission of *Wolbachia* in *Anopheles* mosquitoes (Hughes *et al.*, 2014a). Several other groups have used antibiotics to alter the microbiome and explore the role of microbes in DENV infection in *Ae. aegypti* mosquitoes (Mourya *et al.*, 2002; Hill *et al.*, 2014; Wu *et al.*, 2018) as well as implicate the microbiota in immune pathway activation

in *Ae. aegypti* (Xi *et al.*, 2008; Barletta *et al.*, 2017) and *An. gambiae* (Dong *et al.*, 2009; Kumar *et al.*, 2010). One drawback of this method is that the number and type of bacteria eliminated is generally broad and imprecise, making it difficult to pinpoint specific contributors of the resulting phenotype. In addition, antibiotics can directly affect the host mitochondria, cellular metabolism, alter host immunity and directly enhance viral immunity independent of the microbiome (Stefano *et al.*, 2017; Yang *et al.*, 2017a; Gopinath *et al.*, 2018; VanHook, 2018).

Another approach utilized in microbiome studies is the reintroduction of microbes into mosquitoes by different methods and at various life stages of the mosquito. Microbiome studies involving bacteria supplementation in adult mosquitoes utilize either a physical delivery, like pricking or injection (Dimopoulos *et al.*, 1997; Gorman & Paskewitz, 2000; Schnitger *et al.*, 2007), or orally through blood or sugar meal feeds. *Serratia sp.* have commonly been reintroduced in this way in numerous microbiome related studies as this genus has been implicated in enhancing infection permissiveness to arboviruses including DENV-2 (Apte-Deshpande *et al.*, 2012; Wu *et al.*, 2018) and CHIKV (Apte-Deshpande *et al.*, 2014). *Aeromonas sp.* introduced in a blood meal has also been shown to enhance DENV infection in *Ae. aegypti* (Mourya *et al.*, 2002). A caveat of reintroducing bacteria is understanding the amount needed to be biologically relevant and at what life stage different bacteria are normally acquired, which could affect the dynamics of microbial communities present in the mosquito (Coon *et al.*, 2016; Hegde *et al.*, 2018).

One shortcoming of many vector competence studies is the use of lab strains of mosquitoes (Wilson & Harrup, 2018). Many established lab colonies have been passaged for decades (Kuno, 2010), which could have negative impacts, including reduced adult size and fitness when compared to wild mosquitoes (Ross et al., 2018). Furthermore, variations in rearing temperatures and feeding regimens have been shown to impact the microbiota

and its ability to block pathogens (Moghadam *et al.*, 2017; Guégan *et al.*, 2018) or host susceptibility with regard to malaria infection (Barreaux et al., 2016; Mohanty et al., 2018). Inbreeding has also been utilized to generate lines of mosquitoes that possess specific phenotypes, including reduced sizes, survival rates and lower energy stores (Koenraadt et al., 2010; Ross et al., 2018). Bacterial culture methods and high throughput sequencing (HTS) of the microbiome from field collected and lab reared mosquitoes have concluded that field mosquitoes have a greater bacterial diversity than their lab reared counterparts (Rani et al., 2009; Mwadondo et al., 2017; Hegde et al., 2018).

Here we examined the tripartite interactions between the microbiome and ZIKV in *Ae. aegypti.* To address a number of concerns and unknown variables associated with the microbiome and arboviral infections in artificial settings, we collected wild *Ae. aegypti* mosquitoes from three regions of Texas and included three different lab lines of mosquitoes and exposed them to two American strains of ZIKV; isolate Mex I 7 and clonal Puerto Rico (PR) from the most recent outbreak in 2015. We also explored the impact of ZIKV on the microbiome, by comparing the microbiome of exposed and unexposed mosquitoes. Additionally, we investigated the correlation of total bacterial burden to infection. Finally, we reintroduced bacteria identified by our bioinformatic analyses to assess their impact on vectorial capacity.

METHODS

Mosquitoes

Field mosquitoes were collected from Austin, Brownsville, and Galveston (Texas, USA) over a 3-day period. Mosquito trapping was performed at dusk and dawn using CO_2 baited Fay-Prince traps with collection cups being changed out every hour. Mosquitoes were anesthetized at 4°C and sorted by species and sex. All female *Ae. aegypti* mosquitoes

collected were transferred into cartons, supplemented on 10% sucrose and stored in plastic totes until their arrival at the University of Texas Medical Branch (UTMB) insectary. Adult female *Ae. aegypti* Galveston (F3), Salvador (Brazil; F6) and Rio Grande Valley (RGV; F6) female mosquitoes were reared on 10% sucrose under standard insectary conditions at the UTMB.

Cell Lines and Viruses

Vero cells were maintained in high-glucose Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% fetal bovine serum (FBS) and 1% penicillin/streptomycin at 37°C with 5% CO_2. The viruses used in this study include ZIKV MEX I-7 (KX247632.1) and ZIKV PRVABC-59 (KX377337). Mex I-7 was isolated from the homogenate of an *Ae. aegypti* mosquito on Vero cells with 3 additional passages. All viruses were acquired as lyophilized stocks from the World Reference Center for Emerging Viruses and Arboviruses at the University of Texas Medical Branch (Galveston, TX, USA). Viruses were cultured once in C6/36 *Ae. albopictus* cells, followed by 4 passages in Vero cells to generate stocks for mosquito feeding. ZIKV PRVABC-59 stock was generated from a plasmid clone derived from an isolate from Puerto Rico in 2015 (Yang *et al.*, 2017b). Plasmid linearization, RNA in vitro transcription and electroporation on Vero cells was performed as previously described (Shan *et al.*, 2016). Following electroporation, virus containing supernatant was used to inoculate T-75 flasks of Vero cells for one round of amplification to generate the stock virus (P1).

ZIKV infection and DNA extraction

Both lab and field *Ae. aegypti* mosquitoes were starved for a 24-hour period prior to being offered an artificial blood meal spiked with ~10^6 FFU of ZIKV. For the antibiotic

treatments, groups of 100 mosquitoes (three replicates for each group) were given a sterile 10% sucrose solution with or without 50µg/ml Tetracycline added for three days prior to infection. Mosquitoes were then intrathoracically (IT) injected with ZIKV (Puerto Rico strain, ~500 FFU/mosquito) using a microinjector or fed an artificial blood meal (BM) made with human red blood cells spiked with ~6-logs FFU/ml ZIKV (400µl FBS, 700ul DMEM, 200ul 10% sucrose, 700ul washed red blood cells, 50µl ATP, 4ml virus) and loaded into Hemotek feeding systems (Hemotek Ltd, Blckburn, UK) using naïve mouse skins. Following blood feeding, all non-engorged mosquitoes were removed from each group. Injected mosquitoes were allowed to recover overnight in a humid environment, and both injected and blood fed mosquitoes were maintained on the sucrose solutions with or without tetracycline as indicated for the remainder of the experiment. At 10 days post infection, bodies, legs, and saliva were collected from all surviving mosquitoes to measure infection, dissemination and transmission of the virus, respectively. Only bodies and saliva were collected from IT injected mosquitoes. ZIKV titers were determined by focus forming assay on Vero cells from mosquito samples mechanically homogenized with a TissueLyser II (Qiagen) in 350µl media (DMEM supplemented with 5% FBS, 1% penicillin/streptomycin, and 1% amphotericin). Saliva samples were collected in 5µl FBS for 30 minutes from immobilized mosquitoes before being diluted in 150µl media.

Mosquitoes collected at 10 days post infection were surface sterilized (5 min in 70% ethanol followed by 3 washes in 1X PBS each for 5 min) for DNA analysis following assessment of infection status. To examine both infection and dissemination levels, bodies, and legs were separated into individual tubes and mechanically homogenized in 500µl of tissue culture media. To examine the microbiome composition of ZIKV exposed mosquitoes, total DNA was extracted from 250µl of the body homogenate using the NucleoSpin Tissue kit (Clone Tech) (Hegde *et al.*, 2018).

RNA was isolated from 84 uninfected mosquitoes (45 antibiotic treated, 39 untreated) for gene expression analysis according to the manufacturer's instructions (PureLink RNA Mini Kit, Ambion). From the isolated RNA, cDNA was prepared by digesting 340ng RNA with DNase at room temperature for 15 minutes, followed by an incubation with EDTA at 65°C for 10 minutes. 9µl of DNase treated RNA was used for cDNA synthesis following the manufacturer's protocol (amfiRivert cDNA Synthesis Platinum Master Mix, GenDEPOT).

Estimation of bacterial density

Total bacterial load within each mosquito group was assessed by qPCR using gDNA as a template. qPCR was conducted using universal bacterial primers (Kumar et al., 2010) to amplify the bacterial 16S rRNA gene and a single copy of the mosquito gene S7 (Isoe et al., 2011). The relative abundance of 16S copy number to the endogenous mosquito control was analyzed to determine the total bacterial load in these samples. Cycling conditions and primers are described in (Hegde et al., 2018). The microbial load of each group was estimated from surface sterilized mosquitoes. Mosquitoes were homogenized in PBS and 1/3 of the homogenate was spread onto a plate of LB agar and left to incubate overnight at 37°C. Total prevalence was determined by comparing the overall infected (colonies present) to uninfected (no colonies present) samples.

Focus forming Assay

Clarified mosquito samples and serial dilutions were inoculated onto Vero cells in 48-well plates and overlaid with 0.8% methylcellulose in DMEM. Plates were washed with PBS and fixed with 50:50 methanol:acetone following 4 days of incubation at 37°C. Foci were stained using a mouse hyperimmune polyclonal anti-ZIKV primary antibody (World

Reference Center for Emerging Viruses and Arboviruses, UTMB) and HRP-labeled goat anti-mouse secondary antibody (KPL, Gaithersburg, MD). ZIKV foci were visualized using an aminoethylcarbazole (AEC) detection kit (Enzo Diagnostics, Farmingdale, NY) according to the manufacturer's instructions.

qRT-PCR for Innate Immune Genes

The activation status of the innate immune response was assessed by performing qRT-PCR (SYBR) using RNA prepared from antibiotic treated or untreated mosquitoes. Primers were designed to amplify genes that are part of the major insect innate immune response pathways: Toll, IMD, and JAK-STAT. Relative expression was determined by comparing the ΔCt from the housekeeping S7 gene, and values were transformed as 2-ΔCt to get the fold difference.

Microbiome sequencing and bioinformatics analysis

High-throughput sequencing of the bacterial 16S ribosomal RNA gene has been performed using gDNA isolated from each sample. Sequencing libraries for each isolate have been generated using universal 16S rRNA V3-V4 region primers (Klindworth *et al.*, 2013) in accordance with Illumina 16S rRNA metagenomic sequencing library protocols. The samples were barcoded for multiplexing using Nextera XT Index Kit v2. Sequencing has been performed on an Illumina MiSeq instrument using a MiSeq Reagent Kit v2 (500-cycles). Quality control and taxonomical assignment of the resulting reads was performed using CLC Genomics Workbench 8.0.1 Microbial Genomics Module (http://www.clcbio.com). Low quality reads containing nucleotides with a quality threshold below 0.05 (using the modified Richard Mott algorithm), as well as reads with two or more unknown nucleotides or sequencing adapters were trimmed out. Reference

based OTU selection was performed using the SILVA SSU v128 97% database (Quast et al. 2013). Sequences present in more than one copy but not clustered to the database were then placed into de novo OTUs (97% similarity) and aligned against the reference database with an 80% similarity threshold to assign the "closest" taxonomical name where possible. Chimeras were removed from the dataset if the absolute crossover cost was 3 using a k-mer size of 6. Alpha diversity was measured using Shannon entropy, rarefication sampling without replacement, and with 100,000 replicates at each point. Beta diversity was estimated using D_0.5 Unifrac to show differences in the overall microbiome profiles for antibiotic treated mosquitoes or was calculated using the Bray-Curtis diversity measure.

Statistical Analyses

Statistics were performed in GraphPad (Prism, v7.05). Viral titers were compared by two-tailed Student's t-test and the Kruskal-Wallis test. Prevalence was compared by Chi squared and Fisher's exact test. Alpha diversities were compared by two-tailed Student's t-test and Kruskal-Wallis test.

RESULTS

Microbiota enhance ZIVK infection in mosquitoes.

To investigate the influence of the microbiome on ZIKV infection, we perturbed the microbiome of lab reared *Ae. aegypti* (Salvador strain) with tetracycline and infected the mosquitoes with ZIKV (PRVABC-59) via a blood meal. In contrast to studies that found antibiotic depletion of the microbiome enhanced virus infection for DENV and SINV (Xi *et al.*, 2008; Ramirez *et al.*, 2012; Jupatanakul *et al.*, 2014; Barletta *et al.*, 2017), we observed a significant decrease in ZIKV titers in the bodies of antibiotic treated mosquitoes (Unpaired T test; $p = 0.04$) (Figure 6A). Our findings were similar to a recent study showing that decreasing bacterial load via antibiotic treatment inhibited DENV infection in *Ae. aegypti* (Wu *et al.*, 2018). The titer of disseminated virus, measured as positive ZIKV in the legs of infected mosquitoes, or virus present in saliva was not significantly different between the antibiotic treated and untreated mosquitoes (Figure 6B), nor were there any observed differences in the infection or dissemination prevalence (Figure 7A-B).

In order to bypass the midgut barrier of infection and determine if the microbiota need to be proximal to the pathogen to directly exert an effect, mosquitoes were intrathoracically microinjected with ZIKV (PRVABC-59) (500 FFU/mosquito). Similar to our finding when the virus was administered in the blood meal, we observed a significant reduction in viral titers in the treated mosquito body following a 10-day incubation period (Unpaired T test; $p < 0.0001$) (Figure 6C). No significant differences were seen in virus infection prevalence nor in saliva density or prevalence when comparing antibiotic treated and untreated groups (Figure 7 C-D). The decrease in viral titers when virus is injected into the mosquito suggests that microbiota may alter the virus by modulating the host or by producing molecules that have activity in the hemolymph or fat body.

Antibiotic treatment alters the microbiome of *Aedes aegypti*

We then characterized the microbiome of tetracycline treated and untreated mosquitoes by culture dependent and independent approaches to identify bacteria potentially involved in modulating ZIKV. Although oral administration of tetracycline resulted in non-significant changes in the overall CFU/mosquito ratio, likely due to the large variation seen in individual mosquitoes (Unpaired T test; $p = 0.1$) (Figure 8A), the total number of mosquitoes that possessed culturable bacteria was significantly reduced (Fisher's exact test; $p<0.0001$) (Figure 8B). Similarly, a significant reduction in the total bacterial load in the mosquito was observed when assessed by qPCR (Unpaired T test; $p=0.028$) (Figure 8C).

Given the observed decrease in bacterial load and CFU prevalence in response to oral antibiotic treatment, we assessed the community profile of the two mosquito groups using 16S rRNA amplicon sequencing. After sorting reads to the family level based on 97% similarity clustering of operational taxonomic units (OTUs), we examined the alpha and beta diversities of antibiotic treated and untreated groups using Shannon's Entropy and Unifrac distance comparison, correspondingly. Both alpha diversity and beta diversity displayed significant differences in the overall microbiome profiles, indicating tetracycline treatment profoundly altered the microbiome community composition. Antibiotic treatment resulted in a decrease in alpha diversity (Mann-Whitney test; $p=0.0015$) compared with the untreated mosquitoes. Moreover, the beta diversity plot shows distinct and significant separation of the individual microbiomes for each group (0.5 Unifrac; $p=0.00001$).

The dominant families of bacteria in these lab-reared mosquitoes were *Enterobacteriaceae, Acetobacteraceae, Halomonadaceae,* and *Vibrionaceae* (Figure 10) and it was evident that antibiotic treatment altered microbiome composition. To specifically identify taxa that were significantly altered in response to antibiotic treatment, we performed pairwise comparisons (p=0.05) using ANCOM (Mandal *et al.*, 2015). At the family level, we observed a significant reduction in *Acetobacteraceae* and *Enterobacteriaceae.* (Table 2). Further exploring these findings at the genus level, we found that *Asaia* was the major contributor driving the change in the *Acetobacteraceae* family, while for the *Enterobacteriaceae* family*, Citrobacter, Raoultella, Rosenbergiella,* and an unclassified taxa were significantly different between treatments.

Antibiotic treatment correlates with reduced innate immune gene activation

Modulating the microbiota by antibiotic treatment has been shown to influence mosquito immune pathways that are antagonistic to flaviviruses (Xi *et al.*, 2008; Ramirez *et al.*, 2012). Here we examined and compared the NF-κB transcription activators of the Toll, IMD, and Jak-STAT immune pathways from antibiotic treated and non-treated mosquitoes prior to ZIKV exposure. Similar to the study by Xi *et al*., (2008) we found antibiotic treatment reduced the expression of Rel2, a transcriptional regulator of the IMD pathway, relative to expression in untreated mosquitoes. In addition, the transcription factor STAT, of the Jak/STAT pathway, also exhibited reduced expression in response to tetracycline treatment. Expression of the Toll pathway regulator, Rel1, was also reduced relative to untreated mosquitoes; however, the difference was not significant (Figure 11). In our study we treated mosquitoes with a single antibiotic, tetracycline, whereas Xi et al., (2008) used a cocktail of gentamicin, penicillin and streptomycin to perturb the mosquito microbiome. Despite this difference, we see a similar immune response after depletion of microbiota by antibiotics. This dampening of mosquito immunity, however, does not

adequately explain the reduced viral loads with antibiotic treatment, suggesting the phenotype is mediated by factors other than mosquito immunity.

IMPACT OF THE MICROBIOME ON ZIKV INFECTION IN MOSQUITOES

There are several reports indicating the microbiome of lab-reared and field-caught mosquitoes are significantly different (Rani *et al.*, 2009; Duguma *et al.*, 2015; Mwadondo *et al.*, 2017; Hegde *et al.*, 2018). As such, it is questionable if findings determined in lab-reared mosquitoes translate to the field. Therefore, to examine tripartite interactions in field caught mosquitoes, we trapped host seeking mosquitoes in the field and offered them a ZIKV (MEX 1-7) infectious blood meal. We then correlated their bacterial microbiota and viral infection 10 days post blood meal. Using 16S rRNA amplicon sequencing we explored differences between the microbiome of ZIKV infected and uninfected mosquitoes from each collection site. Using this approach, we recognize that we are only able to capture a snap-shot of the microbiome of these mosquitoes at 10 days post infection (DPI), which may differ from the initial microbiome at the time of infection.

Microbial Diversity and Infection Status

To gauge large scale changes of the microbiome due to infection, we examined the alpha and beta diversity of our different populations. The microbiome of *Ae. aegypti* collected from Austin was significantly different due to infection in terms of both alpha (Mann-Whitney test; $p=0.0077$) and beta (PERMANOVA with Bray-Curtis distance comparison; $p=0.0366$) diversity. No significant changes in either alpha or beta diversity were seen for the mosquitoes collected from Galveston (Mann-Whitney test; $p=0.1179$) PERMANOVA with Bray-Curtis distance comparison; $p=0.0522$) or Brownsville (Mann-Whitney test; $p=0.3357$) PERMANOVA with Bray-Curtis distance comparison; $p=0.9001$)

(Figure 12). Additionally, no differences were seen in the microbiome when comparing mosquitoes with disseminated or non-disseminated ZIKV infection, as measured by FFA of leg homogenates, regardless of mosquito origin (data not shown).

Bacterial Taxa that Contribute to ZIKV Infection Status

The microbiome of all field-collected mosquitoes was dominated by *Asaia*, an *Acetobacteraceae* that commonly infects mosquitoes (Figure 13). In mosquitoes collected from Austin, *Cedecea* were the most prevalent bacteria from this group while in mosquitoes collected from Brownsville and Galveston, *Pseudomonas* and *Serratia* were ubiquitous. When comparing the abundance levels of these bacteria between uninfected, infected, and disseminated mosquitoes, in general we saw a reduction in the relative abundance of *Asaia* in infected and disseminated mosquitoes from Austin and Brownsville (Figure 13A). In contrast, *Cedecea* appeared to expand in relative abundance in the infected and disseminated groups (Figure 13A). However, despite these trends, there was no significant relationship between these taxa and infection status for *Cedecea* (Kruskal-Wallis P =0.32) or *Asaia* in the Austin (Kruskal-Wallis P=0.87) or Brownsville (Kruskal-Wallis P= 0.65) mosquitoes (Figure 13B). This lack of significance is likely attributed to the large variation in the relative abundance of microbiota, which is a common feature of mosquito microbiomes, in addition to the small sample size in the Brownsville group.

To further investigate differentially abundant taxa in each of these groups, we used the ANCOM pairwise statistical analysis, which is specifically designed to handle variable microbiome data. As the test is limited to pairwise comparisons, we compared infected and uninfected mosquitoes. Using this metric, we observed significant increases in fold change at the genus level of ZIKV infected mosquitoes for *Enterobacter, Klebsiella,* and an unknown *Enterobacteriaceae,* all representatives of the *Enterobacteriaceae* family (Table

3). These findings corroborate our data using antibiotic treatment to modulate the microbiome whereby depletion of *Enterobacteriaceae* reduced ZIKV infection. Both Brownsville and Galveston mosquitoes also experienced another singular change in which *Sphingomonas* was elevated in infected mosquitoes compared to uninfected ones.

IMPACT OF ZIKV EXPOSURE ON THE MOSQUITO MICROBIOME

The previous experiment correlated microbiome composition to ZIVK infection. However, from this experiment alone we were unable to determine the direction of the interaction. There is evidence that the microbiota influence infection, but also that infection may alter the microbiome, or possibly both interactions occur simultaneously. To determine how ZIKV exposure and infection alters the microbiome, we compared mosquitoes that imbibed a blood meal to those fed on a ZIKV (MEX 1-7) infected blood meal. The latter cohort were classified into mosquitoes that became infected with the virus or did not. In our Rio Grande Valley (RGV) lab colony (which was collected from a region near Brownsville, TX) we saw that simply exposing these mosquitoes to ZIKV significantly altered their bacterial community (Kruskal-Wallis test; $p=0.037$ and $p=0.001$)(PERMANOVA with Bray-Curtis distance comparison; $p=0.00007$ and $p=0.00001$); however, infection status had no impact on their microbiota composition (PERMANOVA with Bray-Curtis distance comparison; $p=0.797$) (Figure 14A). In contrast to our observations in the RGV lab colony, exposure or infection had a limited effect on the microbiome, with the exception of significant differences comparing the microbial composition of those that were blood fed mosquitoes to those that were ZIKV infected (PERMANOVA with Bray-Curtis distance comparison; $p>0.048$) (figure 14). This suggests that a more robust viral infection is required in the Galveston line to see significant alterations in the microbiota. Furthermore, these findings indicate that alterations of the microbiome in mosquitoes are dependent on the host background, and that differing

responses occur in different host backgrounds. To further explore the impact of ZIKV on the microbiome of the lab strains of mosquitoes, we measured changes in bacterial taxa via ANCOM pairwise analysis (Table 4). In our RGV lab strain we observed that *Asaia* and *Neokomagataea,* both members of the *Acetobacteraceae* family, showed increases in response to ZIKV infection. Similarly, *Neokomagataea* and an unknown *Acetobacteraceae* increased in ZIKV exposed mosquitoes of the Galveston strain. Additionally, *Akkermansia,* a member of the *Verrucomicrobiaceae,* increased in both mosquito lines. *Cedecea,* a member of the *Enterobacteriaceae* family showed a decrease in response to ZIKV infection. Taken together, these results show ZIKV alters the microbiome in a strain specific manner. For certain mosquito strains, exposure to ZIKV alone is able induce these changes, but there are specific bacterial taxa that respond in a similar fashion to viral infection regardless of host background.

As 16S rRNA amplicon sequencing is a relative measure, we sampled the total bacterial load in mosquitoes by qPCR to characterize the interaction between bacterial and viral density. In field caught mosquitoes, we found no differences between ZIKV exposed and infected mosquitoes (Mann-Whitney; $p=0.8702$ Austin, $p=0.9402$ Galveston, $p=0.8404$ Brownsville) (Figure 15). However, for our lab reared mosquitoes, we saw strain specific differences between mosquitoes exposed to ZIKV (including infected) and blood fed mosquitoes. For example, we noted that exposure enhanced infection (Kruskal-Wallis test; $p=0.02$ exposed, $p=0.004$ infected) in the RGV lab strain of mosquitoes (Figure 15). Intriguingly, for both the exposed and infected groups, we noticed a bimodal distribution, which was particularly evident in the infected group (Figure 15). In contrast to our RGV findings, we found that the bacterial load decreased in the Galveston line when mosquitoes were exposed to virus (Kruskal-Wallis test; $p<0.0001$ exposed, $p<0.0001$ infected). For both field-collected and lab reared mosquitoes alike, we saw no differences in the bacterial load when comparing exposed-uninfected to infected. This suggests the microbiome

changes are in response to ZIKV, rather than natural variation in the microbiome influencing ZIKV infection dynamics in the mosquito. To further investigate the intriguing bimodal distribution pattern observed in the RGV strain, we classified mosquitoes into low and high bacterial load groups regardless of infection status and re-analyzed our microbiome data. Here we found no changes in the microbial richness (Mann-Whitney test; $p=0.249$), but we did find significant differences in the composition between these groups (PERMANOVA with Bray-Curtis distance comparison; $p=0.02012$) (Figure 16).

Given that we found an interaction between *Enterobacteriaceae* and ZIKV in lab and field mosquitoes as well as *Acetobacteraceae* in lab mosquitoes, we examined the response of Galveston lab colony mosquitoes (F5) to ZIKV when reinfected at relatively high doses ($2x10^7$) with bacterial isolates belonging to each family via sugar meal. Here we utilized *Asaia,* which tended to increase in the presence of ZIKV and *Cedecea,* which had the opposite response based on our lab infection data. We examined the effect of these bacterial taxa on the infection of two genetically diverse ZIKV isolates, MEX 1-7 and PRVABC59. For mosquitoes infected with the MEX 1-7 strain of ZIKV, supplementation of either *Asaia* or *Cedecea* significantly reduced infection frequencies (Fisher's exact test; $p=0.0092$, $p<0.0001$), but had minimal change on the viral titer of mosquitoes that became infected (Figure 17). *Asaia* also decreased the prevalence of disseminated virus in the mosquitoes (Fisher's exact test; $p=0.0006$) (Figure 17). These bacteria had a more subtle effect on the PRVABC59 clone with *Cedecea* decreasing virus titers compared to the sugar control as well as *Asaia* (Kruskal-Wallis test; $p=0.0456$, $p=0.0455$), but causing no change on overall infection prevalence (Fisher's exact test; $p>0.05$). Similarly, no changes were seen in viral dissemination (Fisher's exact test; $p>0.05$). These virus strains apparently respond differently to the microbiota of their mosquito hosts.

DISCUSSION

While it is clear that complex tripartite interactions influence virus infection of mosquitoes, little is known regarding how ZIKV interacts within the mosquito holobiome. Many studies investigating virus-microbiota interactions use lab strains of mosquitoes. Although mosquito colonies facilitate the ability to carry out these types of studies, their biological relevance for microbiome studies is questionable. In an attempt to overcome this limitation, we used mosquitoes from three field sites in Texas in addition to three separate lines of lab reared colonies to conduct our studies. Additionally, we perturbed the microbiome by different approaches and examined the effect of microbiota on genetically divergent viral strains. The use of different mosquito lines of mosquitoes, both from the field and lab, allow us to validate our findings by highlighting similarities observed between each group. Additionally, the use of two different strains of virus help us to understand the microbiome-virus interaction between different circulating strains of virus.

We found that tetracycline, a bacteriostatic antibiotic, had a major impact on treated mosquitoes. The effects not only impacted the microbiota, but also mosquito susceptibility to ZIKV infection. There are reports indicating that antibiotics can impact cellular metabolism (Yang *et al.*, 2017a; VanHook, 2018), specifically the effects of tetracycline on mitochondria (Ballard & Melvin, 2007; Stefano *et al.*, 2017) and how this disruption can impact viruses in the cell (Ohta & Nishiyama, 2011). However, this may not be the case here, as we also observed decreased viral titers in IT injected mosquitoes, suggesting that this phenotype is independent of the midgut infection. Additionally, regardless of the observed immune dampening in response to antibiotic treatment, we still observed decreased ZIKV titers contrary to previous reports (Mourya *et al.*, 2002; Barletta *et al.*, 2017; Romoli & Gendrin, 2018), suggesting some indirect role of the microbiome on ZIKV susceptibility in mosquitoes (Ramirez *et al.*, 2012; Hegde *et al.*, 2018; Romoli & Gendrin,

2018; Wu *et al.*, 2018). In *An. gambiae,* the antimicrobial effector CEC3 has been shown to increase O'nyong nyong virus (ONNV) infection (Carissimo *et al.*, 2015). Furthermore, this result led to the determination that live microbiota were important for ONNV infection to take place (Carissimo *et al.*, 2015). This suggests that microbiota may alter the virus by producing molecules that have activity in the hemolymph and fat body and can influence other host immune mechanisms or metabolism, which are known to impact arbovirus infection (Xi *et al.*, 2008; Souza-Neto *et al.*, 2009; Behura *et al.*, 2011; Perera *et al.*, 2012; Barletta *et al.*, 2016; Molloy *et al.*, 2016; Jupatanakul *et al.*, 2017).

Microbiota have been shown to have both a positive and negative influence on mosquito borne pathogens (Apte-Deshpande *et al.*, 2012, 2014; Ramirez *et al.*, 2014; Bongio & Lampe, 2015; Wu *et al.*, 2018). In order to determine the role of the microbiome in relation to ZIKV infection, we need to identify potential bacterial candidates and their specific relationship to ZIKV. Given the observed reductions in bacterial loads in tetracycline treated mosquitoes, further examination of the alpha and beta diversity identified the *Acetobacteraceae* and *Enterobacteriaceae* families of bacteria as potential proponents for driving ZIKV susceptibility in these mosquitoes. Deeper analysis identified *Asaia* as the major contributor to the observed differences in the *Acetobacteraceae* family, while the *Enterobacteriaceae* family had a number of contributors in this group.

Asaia is often found to be a predominant member of mosquito microbiomes (Zouache *et al.*, 2011; Osei-Poku *et al.*, 2012; Minard *et al.*, 2013; Hegde *et al.*, 2018; Duguma *et al.*, 2019) and has been characterized as a symbiont of *Anopheles* mosquitoes that can be vertically and horizontally transmitted (Favia *et al.*, 2007; Damiani *et al.*, 2008, 2010; Crotti *et al.*, 2010). *Asaia* has also been shown to be important for mosquito development and inhibition of *Plasmodium* infection (Chouaia *et al.*, 2012; Bongio & Lampe, 2015; Bassene *et al.*, 2018). Here our results suggest that *Asaia* is contributing to

ZIKV infection, similar to Carissimo et al (2015) findings where decreasing microbiota in *Anopheles* lead to reduced ONNV. When *Asaia* was depleted by antibiotics, we observed a decrease in ZIKV regardless of the inoculation route. While the relationship between ZIKV infection and *Enterobacteriaceae* is not as specific, many of the members of this family of bacteria have been described as major contributors to the microbiome in *Aedes* mosquitoes (Ramirez *et al.*, 2012; Coon *et al.*, 2014; Yadav *et al.*, 2016; Dickson *et al.*, 2017a). The idea of *Enterobacteriaceae* impacting ZIKV susceptibility and infection is not that surprising. *Serratia,* a member of the *Enterobacteriaceae* family that was not found to be a major contributor in this study, has been linked to arboviral enhancement in other studies (Apte-Deshpande *et al.*, 2012, 2014; Wu *et al.*, 2018). Together, this opens up the possibility for bacteria in this family to likely have a propensity for enhancing arbovirus infection in mosquitoes. This thought is further supported by our findings from the field collected mosquitoes, specifically the group collected from Austin, where we saw that *Enterobacter* amongst a couple of other taxa from *Enterobacteriaceae* were significantly elevated in infected mosquitoes. Here, our Austin and Brownsville results are in agreement with findings reported by Zink *et al.* (2015), in which they observed relative decreases in bacterial load for certain genera in *Culex* mosquitoes when exposed to West Nile virus (WNV). We observed a relative decrease in *Asaia* and increases in *Cedecea* and *Enterobacter.* While *Asaia* did not experience any significant changes between uninfected, infected or disseminated mosquitoes in our relative abundance or ANCOM analysis in field collected mosquitoes, *Acetobacteraceae* were found to have significant fold increases in response to ZIKV exposure in our RGV and Galveston lab strains. Surprisingly *Acetobacteraceae* bacteria only appear to impact or enhance ZIKV infection in lab reared mosquitoes. It is also notable that *Asaia* in particular is driving these changes, as this genus has been shown to have an inhibitory effect on *P. falciparum* in *Anopheles* mosquitoes (Bassene *et al.*, 2018); however, here we show a propensity for increased arboviral susceptibility.

Given our varied responses to ZIKV infection between different strains of mosquitoes and their microbiomes, we also explored the relationship between total bacterial load to ZIKV infection. Surprisingly, we observed no differences in the number of bacteria present between infected and uninfected mosquitoes. Ramirez and coworkers (2012) had previously demonstrated in *Ae. aegypti* that DENV infection lead to a reduction in bacterial load compared to uninfected mosquitoes (Ramirez *et al.*, 2012), which were ascribed to changes in antimicrobial peptide (AMP) expression levels in response to DENV. AMPs with the ability to interfere with ZIKV infection have been identified (Angleró-Rodríguez *et al.*, 2017; He *et al.*, 2018), and given their antimicrobial properties, it would be expected that these AMPs would have some impact on the mosquito microbiota. However, we observed no changes, suggesting that in our mosquito backgrounds, either the effect of these AMPs was minimal on the microbes present in these mosquitoes or the strains of ZIKV used were unable to stimulate the production of these peptides, further supported by the lack of differences in bacterial loads between disseminated and non-disseminated mosquitoes. Strikingly, the differences we did notice were in comparing ZIKV exposed and unexposed mosquitoes from the lab. At first glance, it appeared that RGV lab mosquitoes had increased bacterial titers when exposed to ZIKV, while Galveston lab mosquitoes presented with decreased levels when exposed. Given these data, it might be possible that these mosquitoes are highly sensitive to ZIKV, and exposure alone is sufficient to activate host responses that can impact the microbiome, such as AMP production.

With regard to the RGV strain, we recognized a bimodal distribution in the bacterial density with one sub-set of the population decreasing in levels similar to the Galveston line, while the second set of the population was in fact increasing in response to exposure. Upon further evaluation of the bimodally distributed bacteria in the RGV line, we found

that there was no difference in species richness between high and low levels of bacteria; however, beta diversity reveled that these microbiomes were indeed distinct from one another. Further analysis would likely help tease out what specific taxa may be driving these stark differences creating the high and low distributions found in the RGV line.

In an attempt to explore the biological impact or relevance of *Acetobacteraceae* and *Enterobacteriaceae* on ZIKV on mosquitoes, we reinfected mosquitoes with isolates of *Asaia* and *Cedecea* followed by ZIKV. We observed conflicting data for each strain of virus used. With regard to the Mex 1-7 isolate, we saw no differences between infection or dissemination viral titers. However, there were significant decreases between both *Asaia* and *Cedecea* infection prevalence when compared to the sugar control group. *Asaia* also led to a decrease in dissemination prevalence when compared to the sugar control group. For the PRVABC59 strain of virus, we saw a subtle, yet significant decrease in the viral titer of mosquitoes treated with *Cedecea* when compared to either the sugar control or *Asaia* treated group. There were no changes in the dissemination titer, nor were there any observed differences in either the infection or dissemination prevalence data. These findings could suggest that there are bacterial strain specific responses to ZIKV, and that the specific isolates used here do not correspond to the responsive taxa that we observed in our ANCOM analysis.

All these data taken together show that *Acetobacteraceae* and *Enterobacteriaceae* are in fact playing some role in enhancing ZIKV infection in *Ae. aegypti* but that these effects could be mosquito-line-specific. Furthermore, specific bacterial isolates could be playing significant roles in mosquito infection and identification of these taxa could potentially require OTU level identification and future characterization.

Table 2. ANCOM Analysis of Differentially Abundant Bacteria in Response to Tetracycline Treatment.

This table outlines the fold change of significantly different responses by bacterial taxa in response to antibiotic treatment $p<0.05$.

Family	Genus	Fold Change
Acetobacteraceae		-2.86
	Asaia	-2.78
Enterobacteriaceae		-2.52
	Citrobacter	-4.66
	Raoultella	-4.77
	Rosenbergiella	-5.48
	Ambiguous_taxa-13	-2.68

Table 3. ANCOM Analysis of Differentially Abundant Bacteria in Response to ZIKV Infection by Location.

This table outlines the observed fold change of significantly different responses by bacterial taxa to ZIKV infection status in mosquitoes from each field location $p<0.05$.

Location	Family	Genus	Fold Change
Austin	*Enterobacteriaceae*	*Enterobacter*	0.95
		Klebsiella	0.76
		Unknown Genus	0.77
Brownsville	*Sphingomonadaceae*	*Sphingomonas*	1.55
Galveston	*Sphingomonadaceae*	*Sphingomonas*	3.01

Table 4. ANCOM Analysis of Differentially Abundant Bacterial in Response to ZIKV Exposure by Lab Line.

This table outlines the fold change of significantly different responses by bacterial taxa to ZIKV infection status in mosquito lines from Galveston and the Rio Grande valley $p<0.05$.

Location	Family	Genus	Fold Change
RGV Lab	*Acetobacteraceae*	*Asaia*	0.10
		Neokomagataea	0.85
	Enterobacteriaceae	*Cedecea*	-7.18
	Oxalobacteraceae	*Janthinobacterium*	-6.15
	Verrucomicrobiaceae	*Akkermansia*	0.20
Galveston Lab	*Acetobacteraceae*	*Neokomagataea*	0.99
		Unknown Genus	0.35
	Bacteroidales S24-7 group	*uncultured bacterium-03*	-4.98
	Erysipelotrichaceae	*Turicibacter*	-4.29
	Flavobacteriaceae	*Elizabethkingia*	7.11
		Flavobacterium	-4.43
	Lactobacillaceae	*Lactobacillus*	-8.41
	Verrucomicrobiaceae	*Akkermansia*	-6.04

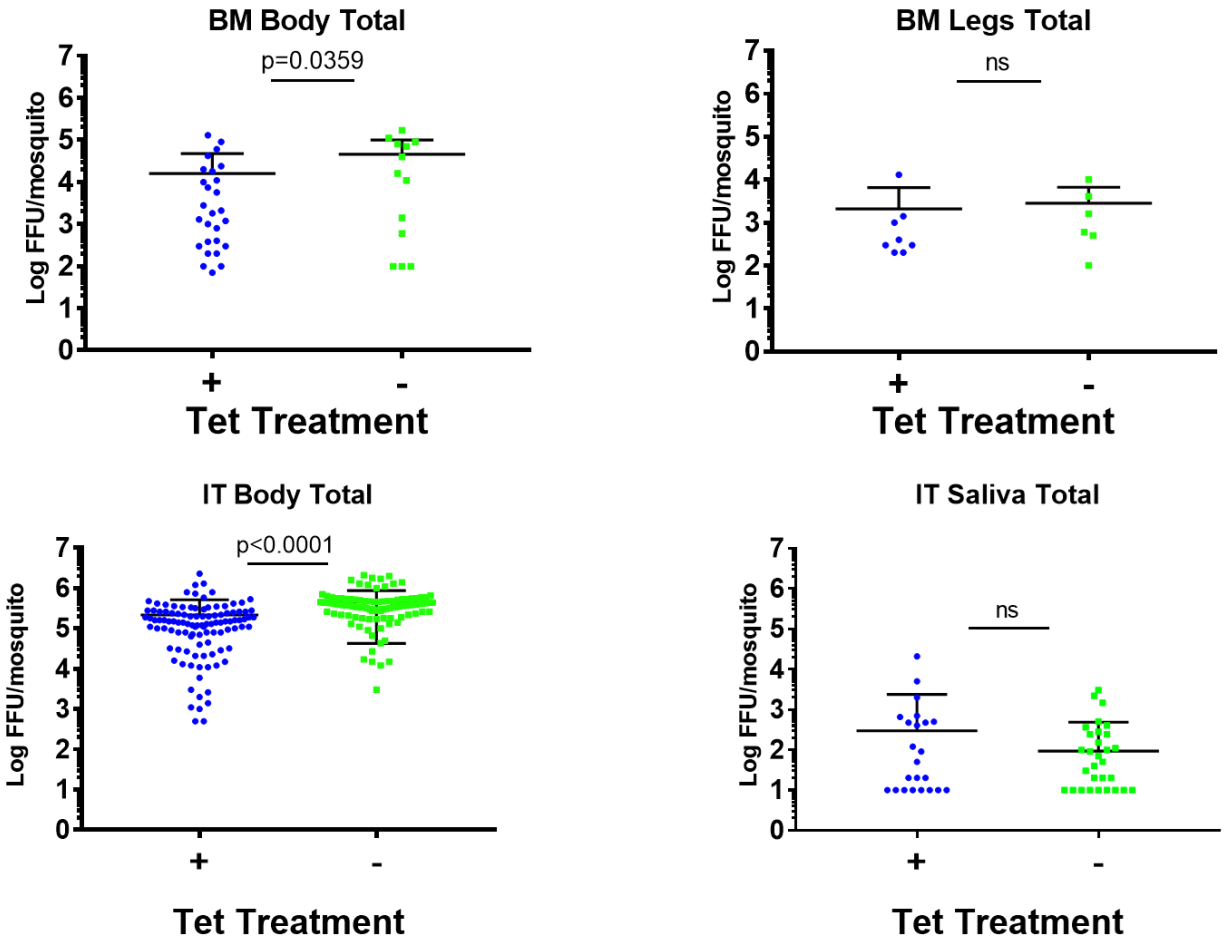

Figure 6. Antibiotic Treatment Reduces ZIKV Infection Titers.

Salvador *Ae. aegypti* mosquitoes were intrathoracically fed a bloodmeal (BM) spiked with ZIKV (PRVABC-59) or (IT) injected with ZIKV following 3 days with or without antibiotic treatment. Titers (ffu/mos.) from infected bodies, legs or saliva samples were determined by serial dilution on Vero cells by focus forming assay. Error bars represent the standard deviation. The mean ZIKV titer in infected mosquito bodies was significantly reduced with antibiotic treatment for both injected and blood fed mosquitoes. Yet, no significant differences in transmission (saliva) or dissemination (legs) mean titers were observed.

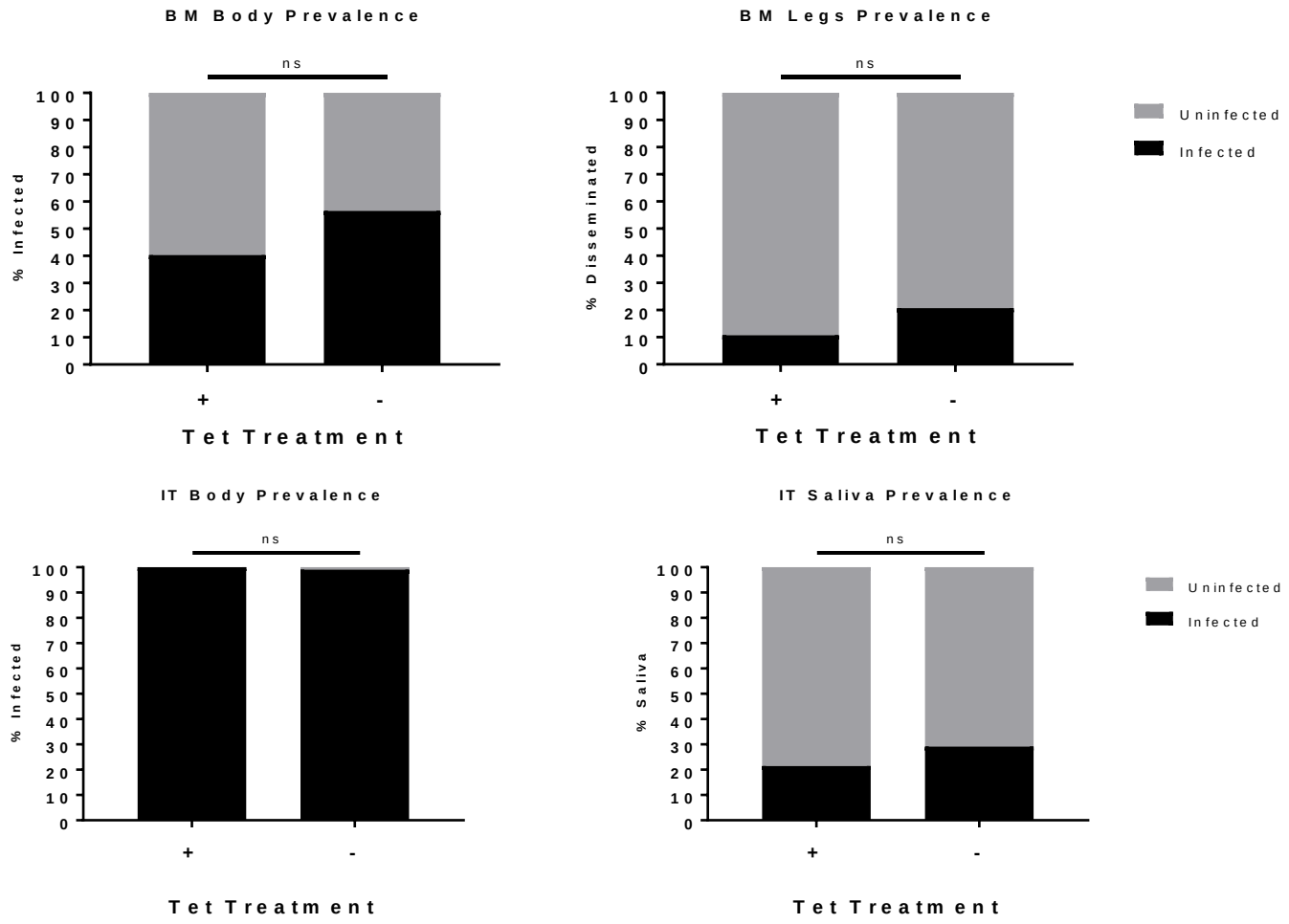

Figure 7. Antibiotic Treatment had no impact on ZIKV Infection Prevalence.

Salvador *Ae. aegypti* mosquitoes were intrathoracically fed a bloodmeal (BM) spiked with ZIKV (PRVABC-59) or (IT) injected with ZIKV following 3 days with or without antibiotic treatment. Titers (ffu/mos.) from infected bodies, legs or saliva samples were determined by serial dilution on Vero cells by focus forming assay. No significant differences in infection (body), transmission (saliva) or dissemination (legs) rates were observed.

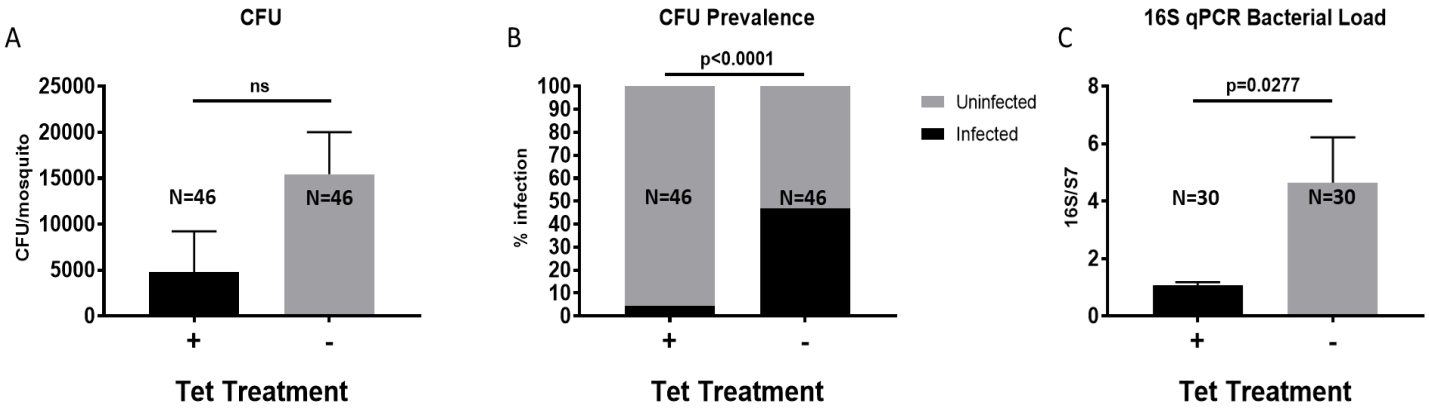

Figure 8. Antibiotic Treatment Decreases the Bacterial Density and Infection Prevalence.

Culturing and qPCR were used to measure differences in the total bacterial load of *Ae. aegypti* following antibiotic treatment. Focus forming assay was used to compare ZIKV infection prevalence between antibiotic treated and non-treated mosquitoes. A-B) Antibiotic treatment greatly reduced the total number of bacteria present in mosquitoes, having a significant impact on the CFU prevalence. C) 16S to S7 ratio measured by qPCR revealed a significant decrease in bacterial load.

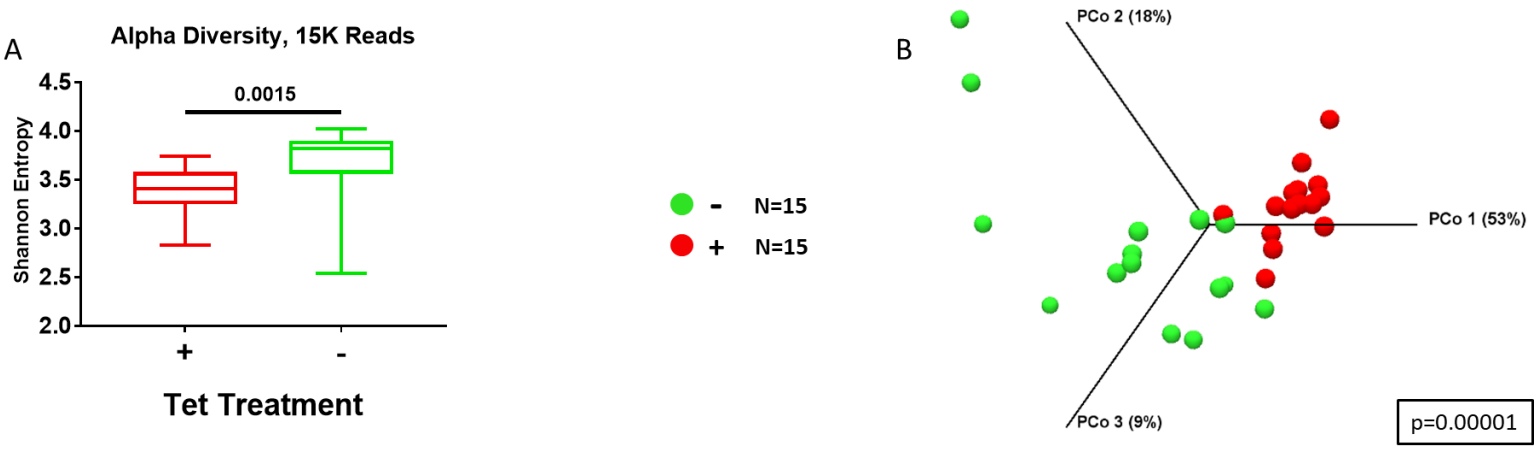

Figure 9. Antibiotic Treatment Alters the Microbiome.

Alpha diversity was calculated by Shannon's Index and beta diversity was calculated using the 0.5 Unifrac diversity measure. A) Antibiotic treatment resulted in a decrease species richness compared to non-treated mosquitoes. B) Additionally, antibiotic treatment lead to a distinct microbial composition between the two groups.

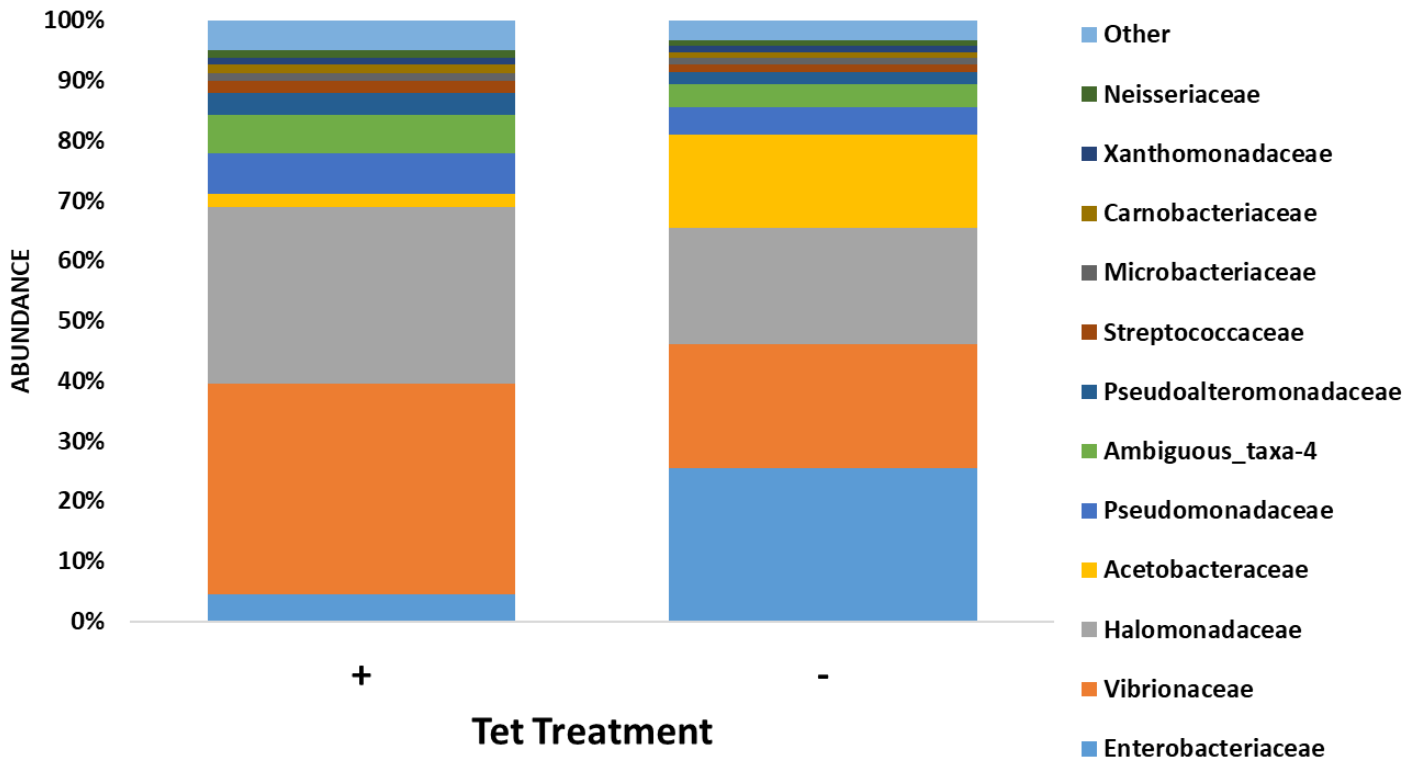

Figure 10. Influence of Antibiotics on Microbial Taxa.

Using relative abundance measurements of the 12 most abundant taxa to assess the impact of Tetracycline on the microbiome. Decreases were specifically noticed for *Enterobacteriaceae* and *Acetobacteraceae* bacterial families in response to antibiotic treatment. *Vibrionaceae, Halomonadaceae, Pseudomonadaceae* and an ambiguous taxa, all appear to fill the void left by decreased bacterial families.

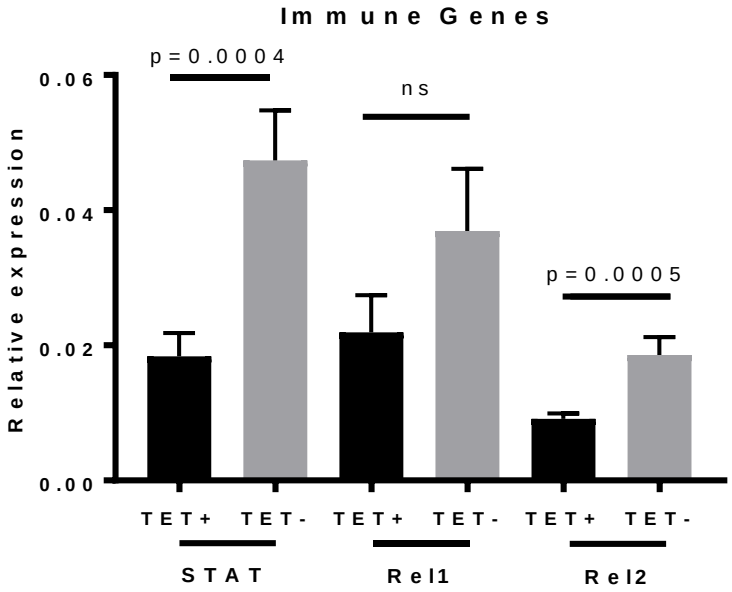

Figure 11. Innate Immune Gene Activation does not Correlate with ZIKV Infectivity.

RNA was isolated from treated or untreated mosquito carcasses, and qRT-PCR was performed using primers for innate immune genes representing the three major pathways in insects. IMD and JAK-STAT pathways were significantly reduced with antibiotic treatment. Error bars represent the standard deviation.

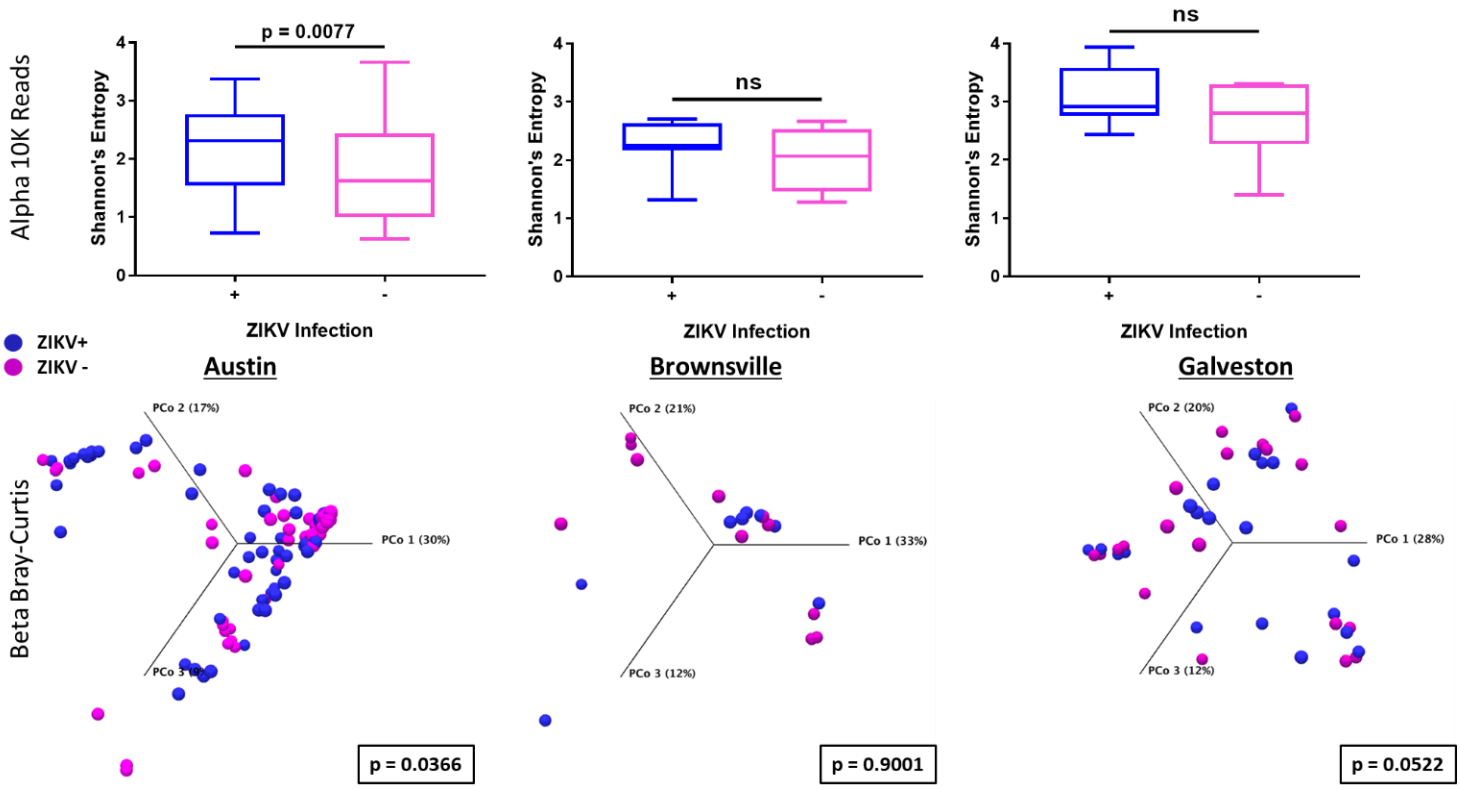

Figure 12. Relationship of Microbiome Diversity to ZIKV Exposure and Infection Status.

Alpha diversity was measured by Shannon's Entropy and differences were calculated by Mann-Whitney test. The three box plots at the top represent the differences in species richness between ZIKV infected and uninfected mosquitoes. Beta diversity was calculated using the Bray-Curtis diversity measure. The three PCoA plots at the bottom represent the bacterial diversity observed between ZIKV infected and uninfected mosquitoes collected from each field site.

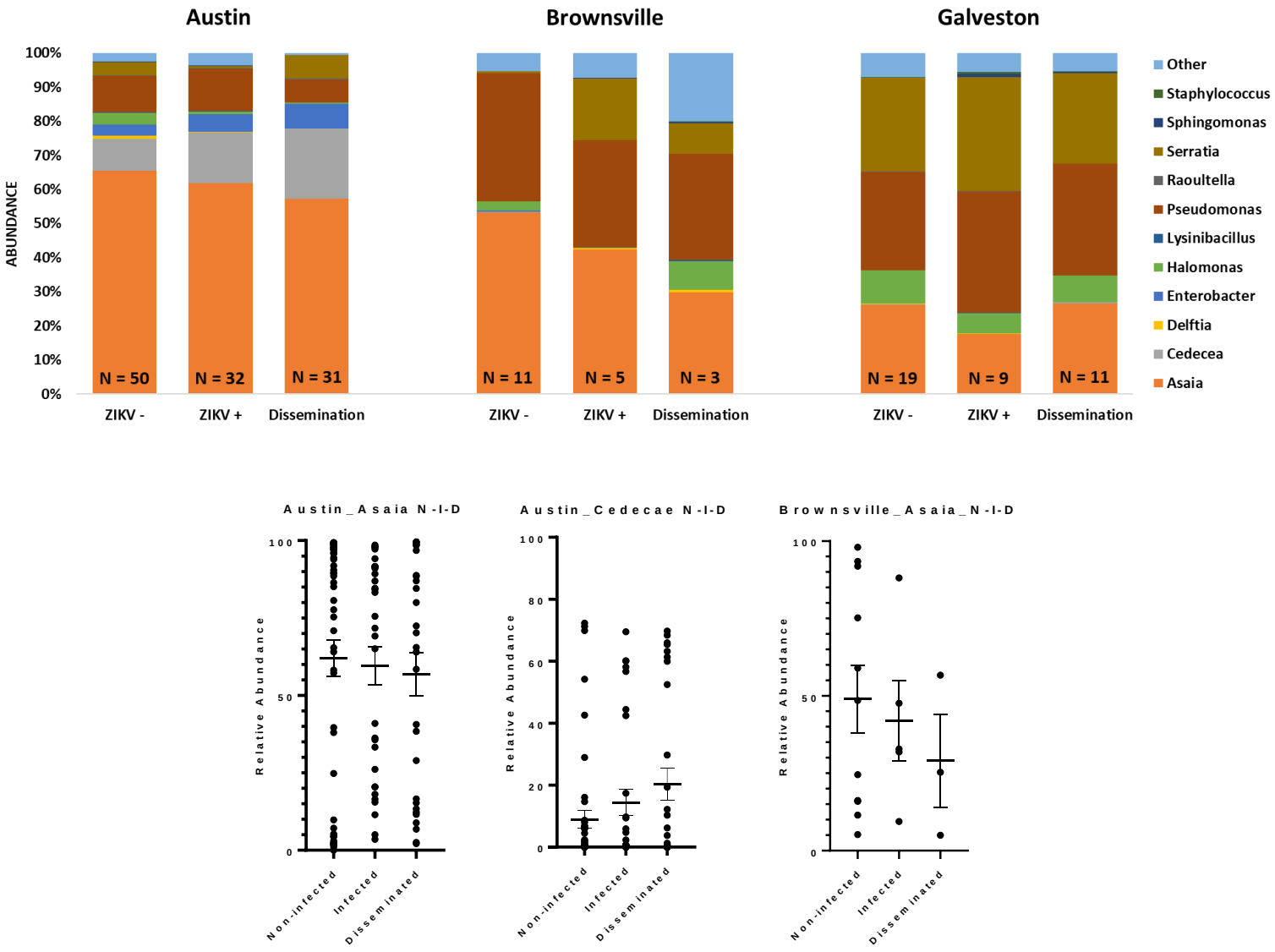

Figure 13. Influence of Microbial Taxa on ZIKV Infection and Dissemination Status.

A) Relative abundance measurements of the most abundant taxa in field collected mosquitoes used to assess their impact on ZIKV infection and dissemination. B) Examination of *Asaia* and *Cedecea* from Austin and Brownsville mosquitoes by infection and dissemination status and measure by Kruskal-Wallis test.

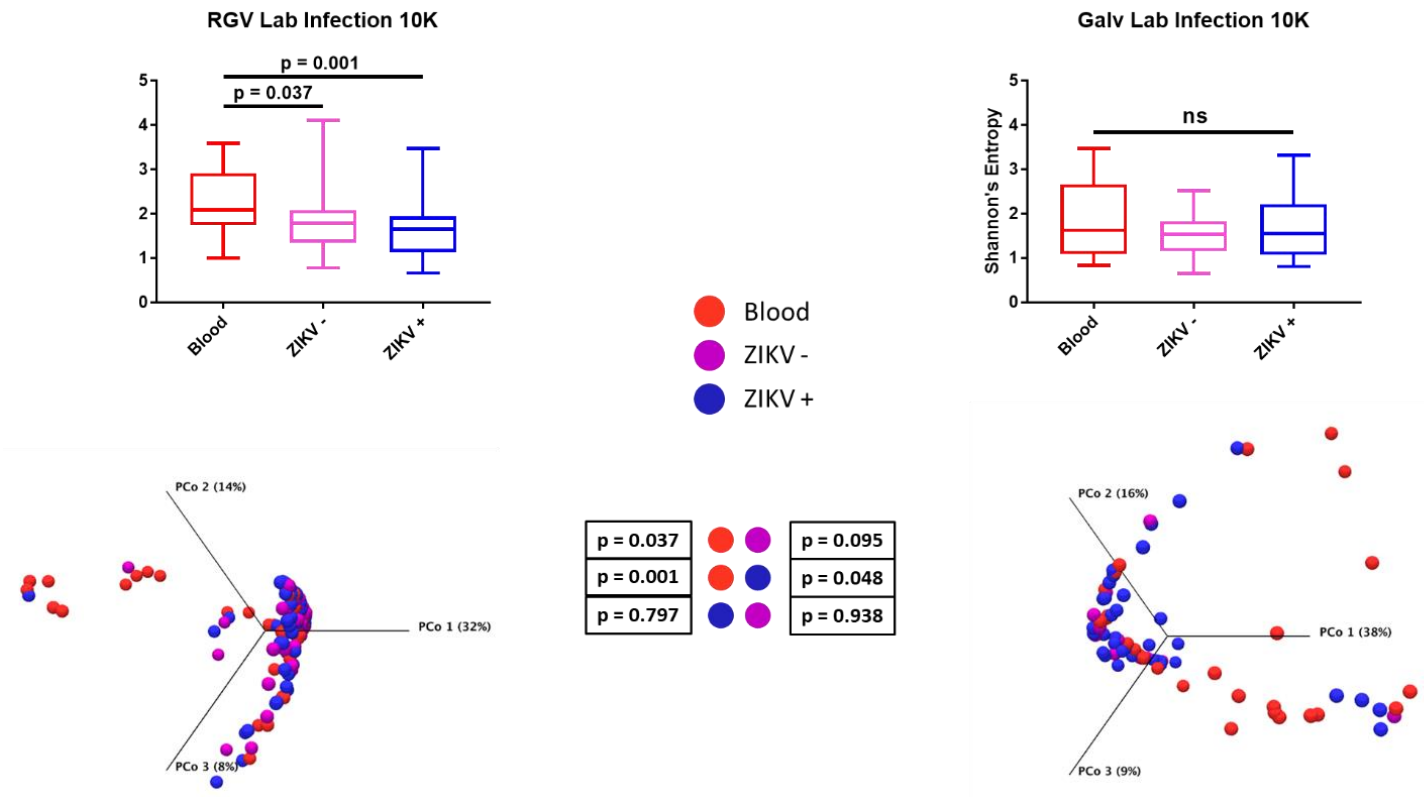

Figure 14. Relationship of Microbiome Diversity to ZIKV Exposure and Infection Status.

Alpha diversity was measured by Shannon's Entropy and differences were calculated by Mann-Whitney test. The two box plots at the top represent the differences in species richness between Blood fed, ZIKV exposed and ZIKV infected mosquitoes. Beta diversity was calculated using the Bray-Curtis diversity measure. The two PCoA plots at the bottom represent the bacterial diversity observed between Blood fed, ZIKV exposed and ZIKV infected lab line mosquitoes.

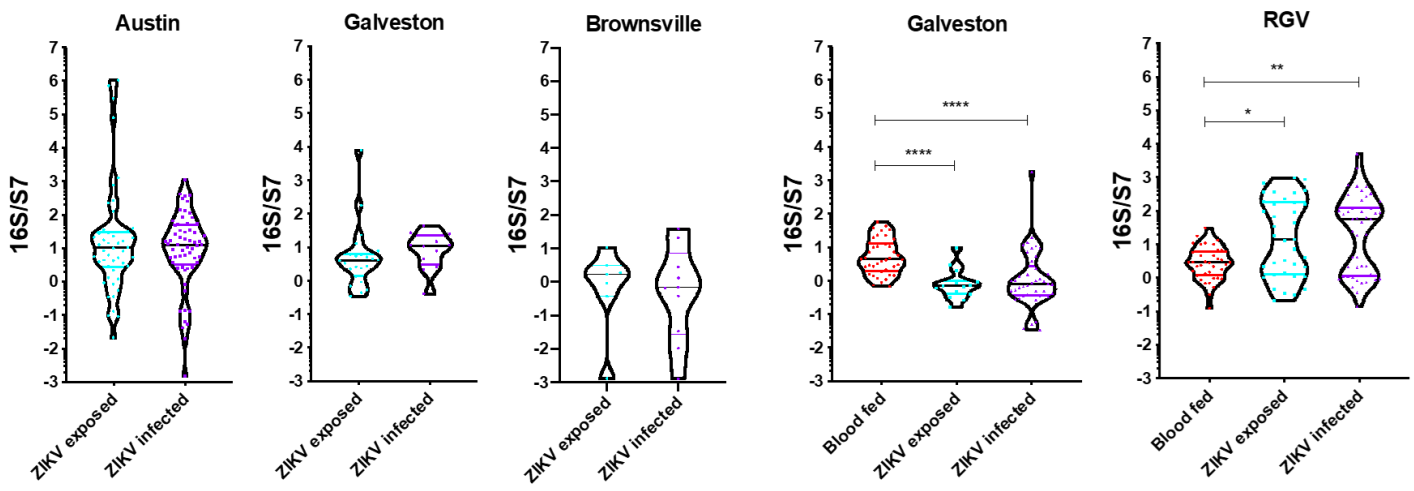

Figure 15. Effect of Total Bacterial Load on ZIKV Exposure and Infection Status.

qPCR was used to measure the ratio of bacteria 16S copies to mosquito S7 copies after 10 days post exposure to ZIKV, to assess if the total load of bacteria has any impact on both exposure and infection status. The three graphs on the left represent the bacterial loads of ZIKV infected and uninfected mosquitoes collected from each field site. The two graphs on the right represent the total bacterial loads of ZIKV unexposed, exposed uninfected and infected lab reared mosquitoes of different origins.

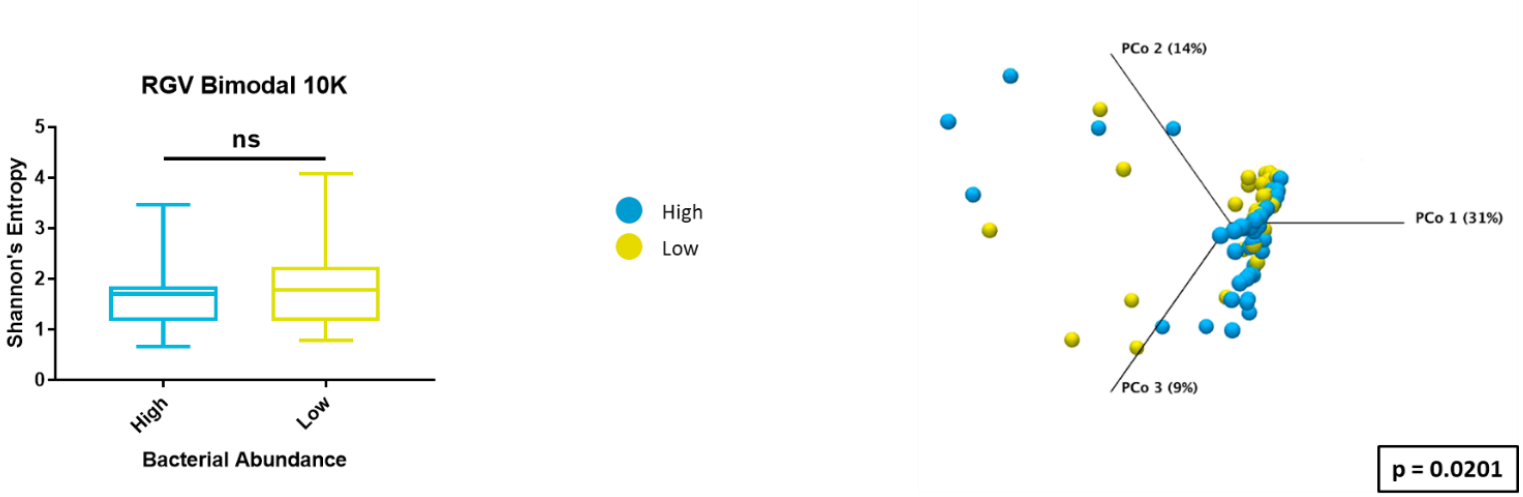

Figure 16. Bimodal Distributed Bacteria have Distinct Microbiomes.

Alpha diversity was measured by Shannon's Entropy and differences were calculated by Mann-Whitney test. The box plot represents the differences in species richness between high and low distributed bacterial loads in RGV mosquitoes. Beta diversity was calculated using the Bray-Curtis diversity measure. The PCoA plot represents the bacterial diversity observed between high and low bacterial loads observed in RGV mosquitoes.

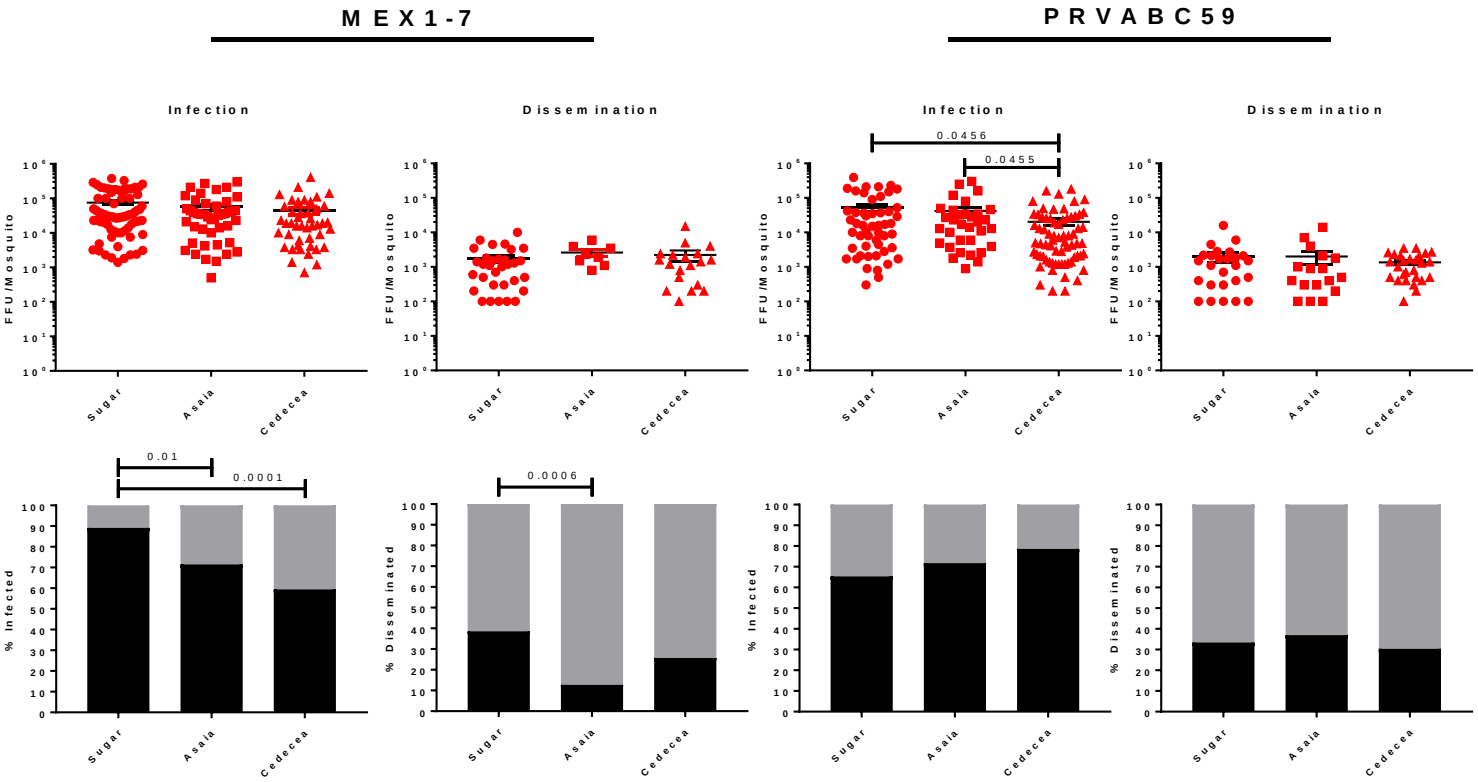

Figure 17. Examination of the biological relevance of bacteria in ZIKV infection by Strain.

Bacteria from the families *Acetobacteraceae (Asaia)* and *Enterobacteriaceae (Cedecea)* were fed to mosquitoes at $2x10^7$ in sugar meals for one week prior to feeding on a ZIKV MX I-7 bloodmeal. Scatter plots at the top represent viral and dissemination titers for Sugar, *Asaia* and *Cedecea* fed mosquitoes. The bar graphs at the bottom represent infection and dissemination prevalence for Sugar, *Asaia* and *Cedecea* fed mosquitoes.

Chapter 4 Zika virus alters the microRNA expression profile and elicits an RNAi response in *Aedes aegypti* mosquitoes[2]

ABSTRACT

Zika virus (ZIKV), a flavivirus transmitted primarily by *Aedes aegypti*, has recently spread globally in an unprecedented fashion, yet we have a poor understanding of host-microbe interactions in this system. To gain insights into the interplay between ZIKV and the mosquito, we sequenced the small RNA profiles in ZIKV-infected and non-infected *Ae*. *aegypti* mosquitoes at 2, 7 and 14 days post-infection. ZIKA induced an RNAi response in the mosquito with virus-derived short interfering RNAs and PIWI-interacting RNAs dramatically increased in abundance post-infection. Further, we found 17 host microRNAs (miRNAs) that were modulated by ZIKV infection at all time points. Strikingly, many of these regulated miRNAs have been reported to have their expression altered by dengue and West Nile viruses, while the response was divergent from that induced by the alphavirus Chikungunya virus in mosquitoes. This suggests that conserved miRNA responses occur within mosquitoes in response to flavivirus infection. This study expands our understanding of ZIKV-vector interactions and provides potential avenues to be further investigated to target ZIKV in the mosquito host.

[2] The work mentioned in this chapter is based on the work published: **Saldaña MA**, Etebari K, Hart CE, Widen SG, Wood TG, Thangamani S, Asgari S, Hughes GL. Zika virus alters the microRNA expression profile and elicits an RNAi response in *Aedes aegypti* mosquitoes. PLoS Negl Trop Dis. 2017 Jul 17;11(7):e0005760. eCollection 2017 Jul. PMID: 28715413. The Creative Commons License can be accessed at http://creativecommons.org/publicdomain/zero/1.0/

INTRODUCTION

Zika virus (ZIKV) is a flavivirus related to dengue virus (DENV), West Nile virus (WNV) and Yellow fever virus (YFV) that is transmitted to humans by *Aedes* mosquitoes. In the urban transmission cycle, *Aedes aegypti* is thought to be the dominant vector, while several *Aedes* species are implicated in transmission in the sylvatic cycle (Diallo *et al.*, 2014; Althouse *et al.*, 2015). The virus was originally discovered in the Ziika forest in Uganda (Dick *et al.*, 1952) and has likely been circulating in monkey and human populations in Africa and Asia. In the last 10 years, an Asian virus lineage has rapidly spread on an unprecedented timescale around the pacific and the Americas. In humans, the neurotropic virus causes microcephaly in newborns and has been implicated in other neurological disorders such as Guillain-Barre syndrome (Rasmussen *et al.*, 2016). The explosive spread of the virus and its effect on infants created a public health emergency and stimulated research efforts to investigate new treatments and vaccines to reduce these conditions. Although significant progress has been achieved concerning the interaction of ZIKV with the mammalian host since the outbreak, we still have a poor understanding of the molecular interplay between the virus and the mosquito host. As vector control is the only viable option for alleviating the diseases caused by ZIKV, a more thorough understanding on these interactions is critical.

Arbovirus infection of mosquitoes elicits complex interactions between the host and the virus. In some cases, the mosquito's innate immune pathways, which can be antagonistic to viral infection, are provoked by arboviruses. However, these immune pathways appear to be virus specific as the Toll and JAK-STAT pathways are antagonistic to DENV yet do not appear to influence other arboviruses such as Chikungunya virus (CHIKV) or ZIKV (Souza-Neto *et al.*, 2009; Ramirez & Dimopoulos, 2010; McFarlane *et al.*, 2014; Jupatanakul *et al.*, 2017). In addition to these classical immune pathways, RNA interference (RNAi) and microRNAs (miRNAs) are important components that dictate

host-microbe interactions for arboviruses and their mosquito vectors (Asgari, 2014; Blair & Olson, 2015; Hussain *et al.*, 2016). PIWI-interacting RNAs (piRNAs), another group of noncoding small RNAs of 25–30 nt, could also potentially be involved in arbovirus-mosquito interactions (Olson & Blair, 2015). miRNAs are small non-coding RNAs (~22 nt) that regulate gene expression post transcriptionally. In mosquitoes, miRNAs are important in many developmental processes and nutrition (Asgari, 2013; Lucas *et al.*, 2013) and it is becoming clear that these molecules are critical in host-pathogen interactions (Asgari, 2011, 2014; Blair & Olson, 2015). Several studies have shown that pathogen infection alters the miRNA expression profile in mosquitoes (reviewed in (Hussain *et al.*, 2016)). This alteration could be due to the host responding to the pathogen or by the pathogen attempting to alter gene expression in the host to make its environment more suitable. For example, the mosquito-borne alphavirus North American eastern equine encephalitis virus (EEEV) alters a host miRNA to avoid the host's immune response (Trobaugh *et al.*, 2014). In *Ae*. *aegypti*, infection with DENV alters the miRNA profile (Campbell *et al.*, 2014), with temporal variation in miRNA expression observed with 23 miRNAs altered at 9 day post infection (dpi) compared to five or less at 2 and 4 dpi. In the Asian tiger mosquito, *Aedes albopictus*, the miRNA, miR-252, increased after a DENV infected blood meal, and inhibition of this miRNA resulted in increased viral copies while overexpression of the miRNA suppressed virus (Yan *et al.*, 2014). Taken together, these studies demonstrate that miRNAs can contribute to the complex interactions occurring between invading arboviral pathogens and their mosquito host, and that this interplay likely dictates vector competence.

While our understanding of these pathways on arbovirus vector competence is expanding, there is a dearth of knowledge related to how ZIKV may alter the miRNA profile in the vector or the human host. To address this issue, here we used high throughput sequencing to examine the small RNA profiles after viral infection of the primary ZIKV

vector *Ae*. *aegypti*. We examined host miRNA, virus-derived short interfering RNA (viRNA) and piRNA profiles at various time points post-infection. Our results provide the first molecular evidence that infection of ZIKV alters the miRNA profile of a host and the mosquito host mounts an RNAi response against the virus.

METHODS

Ethics statement

The ZIKV strain was acquired from the World Reference Center for Emerging Viruses and Arboviruses at the University of Texas Medical Branch (Galveston, TX, USA). The virus was originally isolated from an *Ae*. *aegypti* mosquito (Chiapas State, Mexico). ZIKV protocols were approved by the University of Texas Medical Branch Institutional Biosafety Committee (Reference number: 2016055).

Mosquito infections with Zika virus

Four-six day old female *Ae*. *aegypti* (Galveston strain) mosquitoes were orally infected with ZIKV (Mex 1–7 strain) at 2 x 10^5 focus forming units (FFU)/ml) in a sheep blood meal (Colorado Serum Company). At 2, 7 and 14 days post-infection (dpi) RNA was extracted from whole mosquitoes using the mirVana RNA extraction kit (Life Technologies) following the protocol for extraction of total RNA. Viral infection in mosquitoes was confirmed by Taqman qPCR on ABI StepOnePlus machine (Applied Biosystems) using a ZIKV-specific probe and primers (S4 Table). RNA from ZIKV positive samples was pooled (N = 5) for time points 7 and 14. Limited ZIKV positive samples were detected at day 2, likely due to the virus titer being at the limits of detection for qPCR. For this time point, at least 1 qPCR positive individual was included in each pool. For all time points, three independent pools were used to create libraries for infected

and uninfected samples. Control mosquitoes were fed with blood devoid of ZIKV and collected at the same time points and processed in the same way as infected ones.

Library preparations and sequencing

Small RNA libraries were created using the New England Biolabs small RNA library protocol (New England Biolabs). Library construction used a two-step ligation process to create templates compatible with Illumina based next generation sequence (NGS) analysis. Where appropriate, RNA samples were quantified using a Qubit fluorometric assay (Thermo Fisher Scientific). RNA quality was assessed using a pico-RNA chip on an Agilent 2100 Bioanalyzer (Agilent Technologies). Library creation uses a sequential addition of first a 3' adapter sequence followed by a 5' adapter sequence. A cDNA copy was then synthesized using ProtoScript reverse transcriptase (New England Biolabs) and a primer complimentary to a segment of the 3' adapter. Amplification of the template population was performed in 15 cycles (94˚C for 30 sec; 62˚C for 30 sec; 70˚C for 30 sec) and the amplified templates were PAGE (polyacrylamide gel electrophoresis) purified (147 bp DNA) prior to sequencing. All NGS libraries were indexed. The final concentration of all NGS libraries was determined using a Qubit fluorometric assay and the DNA fragment size of each library was assessed using a DNA 1000 high sensitivity chip and an Agilent 2100 Bioanalyzer. Sequence analysis was performed using the rapid run platform and single end 50 base sequencing by synthesis on an Illumina Hi-Seq 1500 using the TruSeq SBS kit v3.

Small RNA analysis

CLC Genomic Workbench (version 7.5.1) was used to remove adapter sequences and reads with low quality scores from datasets. We applied the quality score of 0.05 as

cut off for trimming. As described in CLC Genomic Workbench manual the program uses the modified Mott trimming algorithm for this purpose. The Phred quality scores (Q), defined as: $Q = -10log_{10}(P)$, where P is the base-calling error probability, can then be used to calculate the error probabilities, which in turn can be used to set the limit for which bases should be trimmed. Reads without 3' adapters or with less than 16 nt were also discarded from the libraries. Clean data were considered as mappable reads for further analysis. We used small RNA tool in CLC Genomic Workbench to extract and count unique small RNA reads with minimum five sampling count. Tab separated files with the read sequences and their counts were used as input file for novel and homologous miRNA analysis using sRNAtoolbox (Rueda *et al.*, 2015). All known *Ae*. *aegypti* precursor miRNAs reported in miRBase 21 were used as reference for miRNA annotation (Kozomara & Griffiths-Jones, 2014). The ultrafast short read aligner Bowtie was used to align the reads to the *Ae*. *aegypti* genome and the miRNA database. The alignment type "n" was selected and we allowed a maximum of one mismatch in the Bowtie seed region for genome, and known and homologous miRNA database in our mapping parameters. The seed alignment length for Bowtie was 20 for all the analyses. Differential expression of miRNAs between two conditions was calculated and normalized based on the DESeq package with EdgeR (Anders & Huber, 2010) on sRNAtoolbox server, and final fold change values were given in $\log_2$ scale.

RNAi activity analysis

To understand the RNAi activity against ZIKV, we mapped all the small RNAs to the viral genome (Accession No. KX247632). We implemented strict mapping criteria (mismatch, insertion and deletion costs: 2: 3: 3, respectively). The minimum similarity and length fraction of 0.9 between a mapped segment and the reference were allowed in mapping criteria. We ignored reads with more than one match to viral genome in mapping

parameters. Mappable reads in all libraries were filtered and only reads with 21 nt in length were selected to check their mapping pattern to negative and positive strands of the virus genome. We also sorted all mappable reads between 25–30 nt to the viral genome for checking any potential piRNA signature.

miRNA target identification

We used three different algorithms including RNA22 (Miranda *et al.*, 2006), miRanda (Enright *et al.*, 2003) and RNAhybrid (Krüger & Rehmsmeier, 2006) to predict potential miRNA binding sites in all the *Ae*. *aegypti* annotated genes (GCF_0000040 15.3_AaegL2) and ZIKV genome (KX247632). The small RNA sequence was hybridized to the best fitting portion of the mRNA or viral genome by RNAhybrid. We did not allow G:U pairing in the seed region (nucleotides 2–8 from the 5' end of the miRNA) and forced miRNA-target duplexes to have a helix in this region. Maximum 5 nt were approved as unpaired nucleotides in either side of an internal loop. miRanda also considers matching along the entire miRNA sequence but we ran the program in strict mode which demands strict 5' seed region (nucleotide 2–8 from the 5' end) pairing. It takes the seed region into account by adding more value to matches in the seed region. RNA22 v2 is a pattern based target prediction program which first searches for reverse complement sites of patterns within a given mRNA sequence and identifies the hot spots. In the next step, the algorithm is searched for miRNAs that are likely to bind to these sites. We allowed maximum 1 mismatch in the seed region and minimum 12 nt matches in the entire binding site. We set the sensitivity and specificity thresholds to 63% and 61%, respectively. miRNA binding sites on *Ae*. *aegypti* mRNAs, which were predicted by all the three algorithms are considered as highly confident miRNA binding sites.

RT-qPCR analysis of miRNAs

RNA samples were converted to cDNA using a miSCRIPT II RT kit (Qiagen) using the HiSpec buffer to assure that the cDNA produced was derived only from mature miRNA molecules. 5μL of RNA was used per reaction with an average 605ng per sample. One additional reaction was prepared with no RNA template. The reaction was heated on a Mastercycler-Pro thermal cycler (Eppendorf). Real-time PCR was performed using an IQ5 cycler (BioRad) and with Quantitech SYBR master mix (Qiagen). The process was performed using the proprietary sequence universal primer provided with the kit as the reverse primer and 10 μM of one of nine miRNA-specific forward primers (IDT), the sequence of which is listed in S4 Table. The cDNA was diluted with 60 μL of nuclease-free water per 30 μL of RT product solution, and 2 μL of diluted cDNA was used per reaction. The volumes of the master mix and primers used were those recommended by their manufacturer. Each sample was run in duplicate and the Ct values averaged for further mathematical processing. The amplification program began with 95˚C for 15min, followed by forty cycles of 94˚C for 15s, 55˚C for 30s, and 70˚C for 30s. Gene expression analysis was performed using the ΔΔCt (Livak) method (Livak & Schmittgen, 2001). The miRNA expression in each sample was normalized to the expression of U6B small nuclear RNA (RNU6B). Our RT-qPCR results confirmed that U6B remained quite stable across infected and noninfected samples (S1 Fig). For each day, six RNA samples were used: three from mock-infected mosquitoes, and three from ZIKV-infected mosquitoes. For each day post-infection, individual ΔCt values for both mock and ZIKV samples were used to calculate relative difference of expression. “No-template” controls were included on each plate run.

Accession numbers

The accession number for the raw and trimmed sequencing data reported in this paper is GEO: GSE97523.

RESULTS AND DISCUSSION

Deep sequencing of small RNAs

Illumina small RNA deep sequencing platform was used to produce small RNA profiles in ZIKV-infected and non-infected *Ae*. *aegypti* mosquitoes. RNA samples were extracted from whole mosquitoes collected at 2, 7 and 14 days post-infection (dpi) to explore host miRNA and RNAi responses to ZIKV infection. ZIKV infection was confirmed in individual mosquitoes by RT-qPCR, which indicated increases in viral load as infection progressed (S2 Fig). We obtained 59.5–61.8 million combined raw reads from the non-infected libraries in day 2, 7 and 14 samples, respectively (S1 Table). From ZIKV-infected libraries, 54.7–84.8 million reads were acquired after combining all the three biological replicates in day 2, 7 and 14 post-infection, respectively (S1 Table). 15–25% of reads were discarded in different libraries due to their low-quality score or lack of adapter sequence. We detected most of the annotated *Ae*. *aegypti* miRNAs present on miRBase in our data representing 10–17% of clean reads in different libraries. In all libraries, total read numbers over different lengths showed a peak at 21–22 nucleotides (nt) representing the typical length of miRNAs and short interfering RNAs (siRNAs) (Fig 18). Another smaller peak at 27–29 nt was obtained probably pertaining to PIWI interacting RNAs (piRNAs), which are common in most insect small RNA libraries.

Differential expression of *Ae*. *aegypti* miRNAs in response to ZIKV infection

Small RNA libraries from ZIKV-infected *Ae*. *aegypti* mosquitoes showed alteration of miRNA profiles compared with non-infected controls at 2, 7 and 14 dpi. However, only 17 miRNAs were identified as differentially modulated at all the time points, with the majority of them significantly depleted in response to ZIKV infection (Table 5). At day 2, 10 *Ae*. *aegypti* miRNAs showed significant changes in their abundance in response to

infection. The maximum fold change (FC) was found in aae-miR-286a, aae-miR-2944b-3p and aae-miR-980-3p with $\log_2$ FC of -1.82, -1.54 and -1.43, respectively (Table 5). Among all the differentially regulated miRNAs, aae-miR-308-3p showed the most considerable depletion (-3.78) at 7 dpi. These values are comparable with miRNA changes seen after DENV infection (Campbell *et al.*, 2014). However, our study and the DENV study (Campbell *et al.*, 2014), sequenced miRNAs using RNA extracted from whole mosquitoes. More pronounced changes are likely to be observed when using specific tissues that are infected with virus. Furthermore, comparison of infected and uninfected tissues may be useful in determining tissue-specific versus systemic changes in miRNAs. Only miRNAs aae-miR-2940-3p, which is mosquito specific, and aae-miR-1-5p were significantly enriched in ZIKV-infected libraries at this time point. We spotted less alteration in miRNA profile at 14 dpi libraries despite mosquitoes at this time point having the highest viral load (S2 Fig). Overall, among all the differentially expressed miRNAs due to ZIKV infection, significant declines in miRNA abundances are more pronounced than enrichment. A similar observation was also reported in a previous study with DENV2, where only 4 miRNAs out of 35 modulated miRNAs during the course of infection were enriched in response to DENV infection (Campbell *et al.*, 2014). Further studies investigating the effect of distinct flaviviruses on miRNA expression in *Aedes* mosquitoes are required to confirm if depletion is a general response to infection. The abundance of a few miRNAs was altered in more than one time point after ZIKV infection including, aae-miR309a, aae-miR-308, aae-miR-286b, aae-miR-2941 and aae-miR-989.

To validate the differentially expressed miRNAs, nine miRNAs were selected. For this, RNA samples extracted from non-infected and ZIKV-infected whole mosquitoes at 2, 7 and 14 dpi were subjected to miRNA-specific RT-qPCR. Our results showed broad agreement between qPCR and NGS values. While it is not uncommon to find inconsistences between these two quantification approaches (Liu *et al.*, 2015; Li *et al.*,

2016a), in 18 out of 27 cases, the direction of gene expression was the same (i.e. both enriched or both depleted) (Fig 19). Where discrepancies were observed, the trend was for NGS data to indicate depletion of the miRNA, while the qPCR suggested no significant changes. A notable inconsistency was seen with the miRNA miR-308-3p that was seen to be enriched by qPCR but depleted by deep sequencing at 7dpi.

A cell line study using *Ae*. *aegypti* Aag2 cells found miRNAs were only mildly affected by DENV infection (Miesen *et al.*, 2016), but in contrast a number of mosquito studies, reported differentially abundant miRNAs in response to a number of arboviruses. However, in most cases, follow up studies to explore the functional significance of those changes and effects on host target genes and virus replication are lacking. Therefore, below we mainly compare the miRNA changes identified in our study with those in previous ones. In *Ae*. *aegypti* mosquitoes infected with DENV2, five, three and 23 miRNAs were differentially expressed at 2, 4 and 9 dpi, respectively (Campbell *et al.*, 2014). Among those, miR-308-3p and miR-305-5p (9dpi) overlap with those in ZIKV-infected mosquitoes at 7 and 14 dpi; in both host-virus systems both miRNAs showed depletion. In *Ae*. *albopictus* DENV2-infected mosquitoes, overlapping differentially abundant miRNAs with ZIKV-infected mosquitoes from this study are miR-2940-3p (depleted in DENV, but enriched in ZIKV), miR-263a-5p (depleted in both), miR-308-5p (enriched in both), miR-989 (depleted in DENV, but enriched in ZIKV), and miR-2941 (depleted in both) (Liu *et al.*, 2015). In another study from the same group with *Ae*. *albopictus* and DENV2 infection specifically in the midgut tissue, three miRNAs (miR-2941, miR-989, miR-2943) were differentially expressed (Liu *et al.*, 2016b), the first two also with change in abundance upon ZIKV infection in this study. Furthermore, miR-989 was found to be depleted in *Culex quinquefasciatus* mosquitoes by 2.8-fold when infected with WNV (Skalsky *et al.*, 2010); although this miRNA was enriched by about 1.8-fold at 2 and 14 dpi with ZIKV in

the present study. miR-980 was also differentially expressed in the *Cx*. *quinquefasciatus*-WNV interaction (Miranda *et al.*, 2006).

It appears that the identified differentially expressed miRNAs in different host mosquitoes upon flavivirus infections overlap more with each other than infections with other viruses, such as alphaviruses. For example, none of the major *Ae*. *albopictus* miRNAs that were differentially abundant after CHIKV infection (miR-100, miR-283, miR-305-3p, miR-927) (Shrinet *et al.*, 2014) were found among the list of differentially expressed miRNAs from this study; although some of the differentially expressed miRNAs as a result of ZIKV infection could be found among miRNAs showing low levels of differential expression in the CHIKV-mosquito interaction. The similarities in miRNA changes in mosquitoes when infected with flaviviruses as compared to alphavirus infections could be due to (1) antigenic differences between flaviviruses and alphaviruses that may elicit slightly different host responses, or (2) differences in replication strategies; for example, production of subgenomic flavivirus RNA (sfRNA) by flaviviruses, which could function as decoys or sponges against host derived miRNA, suppress the RNAi response, and play other important roles in mosquito-virus interaction (Schnettler *et al.*, 2012; Pijlman, 2014; Roby *et al.*, 2014). Interestingly, sfRNA from WNV has been shown to efficiently suppress siRNA and miRNA-induced RNAi pathways in mosquito cells and its engineering into a Semliki Forest virus (SFV, an alphavirus) replicon led to enhanced replication of SFV in RNAi-competent mosquito cells (Schnettler *et al.*, 2012). While alphaviruses do not produce such RNAs and must rely on other mechanisms to deregulate the host RNAi response.

Target analysis of differentially abundant miRNAs

The hypothetical binding sites for all the differentially abundant miRNAs upon ZIKV infection were predicted by command line tools miRanda, RNAhybrid and RNA22 v2 using their default parameters. High confidence potential targets were defined as those containing a unique binding site for each miRNA in all the algorithms, with a maximum of 10 nucleotides shifting. We predicted 898 mRNAs, which can potentially be regulated by the differentially abundant miRNAs upon ZIKV infection (S2 Table). Among these predicted target genes, 247 binding sites were identified for aae-miRNA-980-3p while only six predicted binding sites were detected for aae-miR-308-3p. Although this miRNA showed more profound regulation in response to viral infection (day 7), we only identified Rho GTPase as its predicted target gene (S2 Table). Other predicted binding sites for this miRNA are located on coding regions of some hypothetical proteins. Rho proteins are small signaling G proteins, which are involved in a wide range of cellular functions such as cell polarity, vesicular trafficking, the cell cycle and transcriptome dynamics (Bustelo *et al.*, 2007). Among the predicted targets, a number of immune-related genes were found, such as leucine-rich immune protein and Toll-like receptor, possibly indicating the ability of ZIKV to modulate mosquito immunity. While the list of targets provides a catalogue of high confidence targets of *Ae*. *aegypti* differentially abundant miRNAs upon ZIKV infection, further investigations are required to experimentally establish miRNA-target interactions.

Whilst miRNA-target studies have not been carried out on any of the miRNAs reported to be differentially abundant following viral infection in mosquitoes (previous section), except aae-miR-2940-5p, the role of some of these miRNAs are known in other aspects of mosquito or *Drosophila* biology. For example, a number of the differentially expressed miRNAs upon ZIKV infection were also found differentially expressed upon blood feeding in the fat body tissue (Zhang *et al.*, 2016). These include, miR-308-5p, miR-263a-5p, miR-305-5p, miR-989, miR-2941, miR-286b, miR-2946. miR-309a, specifically

was shown to control ovarian development by targeting the Homeobox gene SIX4 (Zhang *et al.*, 2016), and miR-375 was found highly induced in blood fed mosquitoes regulating a number of mosquito genes, including upregulating *cactus* and downregulating *Rel1* (Hussain *et al.*, 2013). Application of miR-375 mimic in Aag2 cells led to enhanced DENV replication. While this miRNA was found to be mostly depleted after ZIKV infection (Fig 19), it will be interesting to experimentally test if manipulation of this miRNA could have any effect on ZIKV infection by regulating the Toll pathway. In *D. melanogaster*, the role of miR-308 in development (Daneshvar *et al.*, 2013), miR-980 in memory (Busto *et al.*, 2015), and miR-305 in homeostasis (Foronda *et al.*, 2014) have been reported.

We also screened the ZIKV genome for potential miRNA binding sites of all the 17 modulated miRNAs. Eighty-five possible interactions were identified by three different target predicting algorithms (miRanda, RNAhybrid and RNA22). S3 Table summarizes highly confident binding sites that were predicted by more than one tool. Some miRNAs such as aae-miR263a5p, aae-miR-286, aae-miR-305-5p, aae-miR308-5p, aae-miR-989 and aae-miR-980-3p can potentially bind to more than one place in the viral genome. Previously, targeting of genomes of RNA viruses by host miRNAs have been reported in mammalian cells (Ahluwalia *et al.*, 2008). In particular, a number of human miRNAs (hsa-miR-133a, hsa-miR-548g-3p, hsa-miR-223) with potential binding sites in the 5' and 3'UTRs of different DENV serotypes have been shown to negatively affect replication of the viruses when overexpressed in mammalian cells (Wen *et al.*, 2015; Castillo *et al.*, 2016). In mosquitoes, a midgut-specific alb-miR-281 from *Ae. albopictus* was shown to target the 5'UTR of DENV2 thereby enhancing replication of the virus (Zhou *et al.*, 2014).

ZIKV is a target of the *Ae. aegypti* RNAi response

Flaviviruses generally produce dsRNA intermediates during their replication, which are the target of their invertebrate host RNAi machinery (Blair & Olson, 2015). The long dsRNAs are recognized and subsequently diced by the ribonuclease Dicer-2 into 21 nt virus-derived short interfering RNAs (viRNAs) that are double stranded and induce the formation of the RNA induced silencing complex (RISC). One of the strands of the duplex is degraded and the other one guides the RISC complex to viral target sequences with complete complementarity. This binding results in the cleavage and degradation of viral RNAs produced during replication of the virus.

To investigate potential RNAi activity against ZIKV, we mapped all the small RNAs to the viral genome (accession no. KX247632). In total, 3,288, 20,360 and 57,867 reads mapped to the viral genome at 2, 7 and 14 dpi, respectively, ranging in size from 15–35 nt. The total number of reads at 14 dpi that mapped to the virus genome accounted for 0.16% of the total small RNA reads at this time point after infection (36,115,068; S1 Table), which is close to the percentage (0.05%) found in DENV2-infected *Ae*. *aegypti* whole mosquitoes at 9 dpi (Scott *et al.*, 2010). The number could possibly be higher if small RNAs are analyzed in specific tissues where virus infection primarily occurs. Using whole mosquitoes, which is a mixture of infected and non-infected tissues, may result in dampening of the percentage of virus-specific small RNAs. While at 2 dpi the distribution of small RNAs was across different sizes, at 7 and 14 dpi the majority of the mapped reads were at 21 nt, typical of viRNA size in mosquitoes (Fig 20A). When only the 21 nt reads were mapped to the viral genome, the number of viRNAs increased dramatically during the course of infection; 201 (2 days), 6,250 (7 days), and 20,732 (14 days). This also confirmed successful replication of the virus in the mosquitoes. In addition, the viRNAs mapped across the entire length of the viral genome, on both positive and negative strands of the viral genome (Fig 20B). The pattern of mapped reads indicated a bias towards the positive strand; 62% to the positive strand and 38% to the negative strand–the percentages

were very similar both at 7 and 14 dpi. We did not find distinct hot-spots (large number of viRNA production) across the viral genome, except one towards the end of the NS5 region at both 2 dpi and 7 dpi, which is also present at 14 dpi but not as a pronounced peak among others (Fig 20B). These results confirm that ZIKV is exposed to the mosquito host RNAi response, with the replicative dsRNA intermediates being the major substrate for Dicer-2. These findings are consistent with other examples of flaviviruses (Brackney *et al.*, 2009, 2010; Scott *et al.*, 2010; Hess *et al.*, 2011; Aguiar *et al.*, 2016; Göertz *et al.*, 2016; Miesen *et al.*, 2016).

Production of ZIKV-derived piRNA-like small RNAs

Virus-derived piRNA-like small RNAs (25–30 nt), which are also referred to as viral-derived piRNAs (vpiRNAs), have been identified in insects infected with flavivirues, bunyaviruses and alphaviruses (Scott *et al.*, 2010; Morazzani *et al.*, 2012; Vodovar *et al.*, 2012; Schnettler *et al.*, 2013a, 2013b). It has been shown that knockdown of the piRNA pathway proteins leads to enhanced replication of arboviruses in mosquito cells, suggesting their potential antiviral properties in mosquitoes. For example, knockdown of Piwi-4 in *Ae. aegypti* Aag2 cell line increased replication of the mosquito-borne alphavirus, SFV (Schnettler *et al.*, 2013a). In another study in the same cell line, specifically silencing Ago3 and Piwi-5 led to significantly reduced production of vpiRNAs against another alphavirus, Sindbis virus (SINV) (Miesen *et al.*, 2015).

To find out if any virus-derived piRNA-like small RNAs are produced in *Ae. aegypti* mosquitoes infected with ZIKV, we mapped 25–30 nt small RNA reads from the three time points post-infection to the viral genome. The number of reads increased as infection progressed, and they mapped to the entire ZIKV genome with no particular hot spots identified (Fig 21). However, we found a significant bias for reads mapped to the

positive strand; for example, in 14 dpi samples 5,300 of 25–30 nt reads mapped to the positive stand and only 60 reads mapped to the negative strand (Fig 21). In DENV2 infected *Ae*. *aegypti* mosquitoes, the number of 25–30 nt reads that mapped to the negative strand of the virus were also extremely low. Further, no bias for a specific base or sequence-specific piRNA signature (U_1 and A_{10} bias) was observed in this study, as would normally be expected for ping-pong derived piRNAs (Brennecke *et al.*, 2007).

Similar observations were reported in other flavivirus-infected mosquitoes or mosquito cell lines. We recently demonstrated that in *Ae*. *aegypti* mosquitoes infected with an insect-specific flavivirus (Palm Creek virus), small RNA reads in the range of 25–30 nt do not harbor any of the classical sequence-specific piRNA features (Lee *et al.*, 2017). Hess et al. (2011) also showed that DENV2 piRNA-like sequences do not display any bias for U in the first position and only a slight bias for A_{10} (Hess *et al.*, 2011). However, in mosquito cells infected with alphaviruses SFV (Schnettler *et al.*, 2013a) and SINV (Vodovar *et al.*, 2012), and bunyaviruses La Crosse virus (Vodovar *et al.*, 2012) and Rift Valley Fever virus (Dietrich *et al.*, 2017) clear U_1 and A_{10} pingpong piRNA signature was observed. Hence, currently we do not have enough evidence to classify the 25–30 nt reads that mapped to the ZIKV genome as vpiRNA since they might be artefacts of viral genome degradation.

In summary, we found that ZIKV infection in *Ae*. *aegypti* altered the small RNA profile of mosquitoes with peaks seen at 21–22 and 27–29 nt. Overall, ZIKV infection modulated 17 miRNAs with the majority of these small RNAs being depleted. Several immune related transcripts were the predicted targets of differentially abundant miRNAs suggesting that ZIKV may interact with mosquito immunity. At 7 and 14 dpi, viral infection initiated an RNAi response indicated by the presence of viRNAs. At these times points, virus-derived small RNAs in the size range of piRNAs were also found in infected

mosquitoes, although they lacked the typical piRNA signature. This study increases our understanding of ZIKV-mosquito interactions and broadens our comprehension of the *Aedes* miRNA response to flavivirus infection.

Table 5. Differentially expressed *Ae. aegypti* miRNAs upon ZIKV infection.

	Normalized RC (Control)			Normalized RC (ZIKV infected)					
	R1	**R2**	**R3**	**R1**	**R2**	**R3**	**Log FC**	**P-Value**	**FDR**
Day 2									
miR-263a-5p	36652.15	27181.08	22225.01	19050.03	12791.09	18564.69	-0.772	>0.001	0.010
miR-286a	152.99	113.75	71.87	24.82	11.92	58.29	-1.824	>0.001	0.003
miR-2941	28750.73	29156.66	24196.24	16038.26	14054.41	20628.86	-0.695	0.001	0.011
miR-2944b-3p	728.56	582.92	534.37	233.93	68.96	330.77	-1.542	>0.001	0.009
miR-2944b-5p	9117.94	6211.78	5055.22	3142.95	1189.77	4121.39	-1.270	>0.001	0.009
miR-2946	6408.73	7195.53	5317.72	3947.63	3808.98	4286.01	-0.652	>0.001	0.010
miR-308-5p	533.90	590.62	634.02	1092.08	1236.04	734.92	0.800	0.002	0.034
miR-309a	1617.69	1269.01	1487.04	573.99	242.64	879.29	-1.366	>0.001	0.008
miR-980-3p	54.66	36.44	53.25	16.71	16.41	19.90	-1.431	>0.001	0.003
miR-989	22085.75	47068.61	43795.06	76596.29	88415.35	45462.50	0.898	0.002	0.034
Day 7									
miR-286b	361.26	511.03	611.97	326.68	147.02	173.10	-1.198	>0.001	0.008
miR-2940-3p	15150.75	22040.64	17096.52	32250.97	28203.91	35773.85	0.826	>0.001	0.004
miR-2941	40698.57	37427.95	44481.30	31999.67	18418.53	21111.91	-0.777	>0.001	0.014
miR-308-3p	58.77	100.97	350.85	9.03	18.25	9.51	-3.781	>0.001	0.000
miR-308-5p	1338.45	1111.09	1030.21	766.07	772.37	758.71	-0.599	0.001	0.018
miR-375	2793.59	2960.56	3169.38	2186.33	1741.64	1855.77	-0.626	>0.001	0.014
mir-1-5p	7.46	0.29	5.87	17.41	11.71	34.06	2.148	0.002	0.035
Day 14									
miR-286b	438.08	547.87	308.77	202.32	276.78	193.60	-0.939	>0.001	0.007
miR-305-5p	8139.34	10259.61	7866.41	6668.66	6079.73	5420.66	-0.531	0.002	0.036
miR-308-3p	196.88	110.89	75.05	28.07	47.80	29.08	-1.862	>0.001	0.000
miR-309a	169.84	200.41	112.85	74.58	65.35	27.69	-1.514	>0.001	0.001
miR-71-5p	1538.88	1539.48	1447.11	1780.10	2389.32	2752.36	0.612	>0.001	0.012
miR-989	76630.42	62358.80	77297.98	108619.53	113002.20	156294.84	0.805	>0.001	0.001

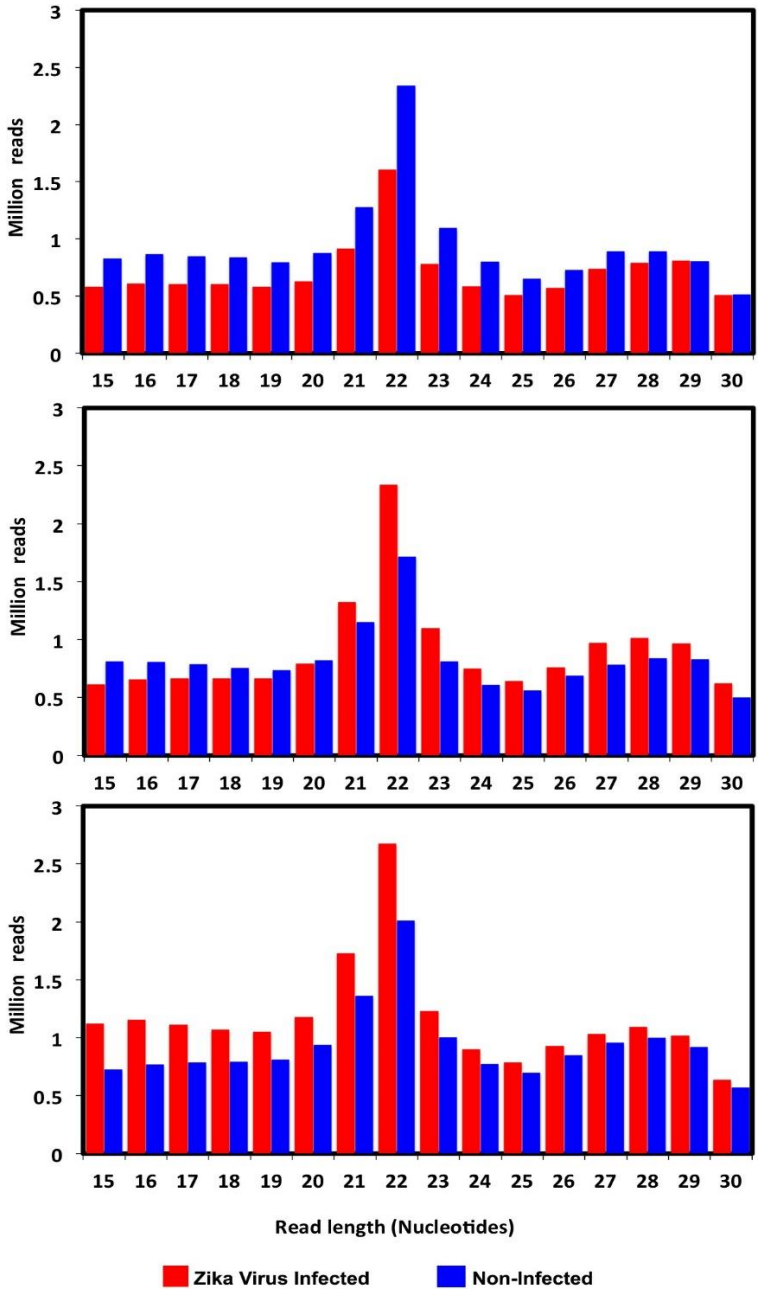

Fig 18. Length distribution of mappable reads to the *Ae. aegypti* genome obtained from ZIKV-infected and non-infected mosquitoes at day 2, 7 and 14 post-inoculation.

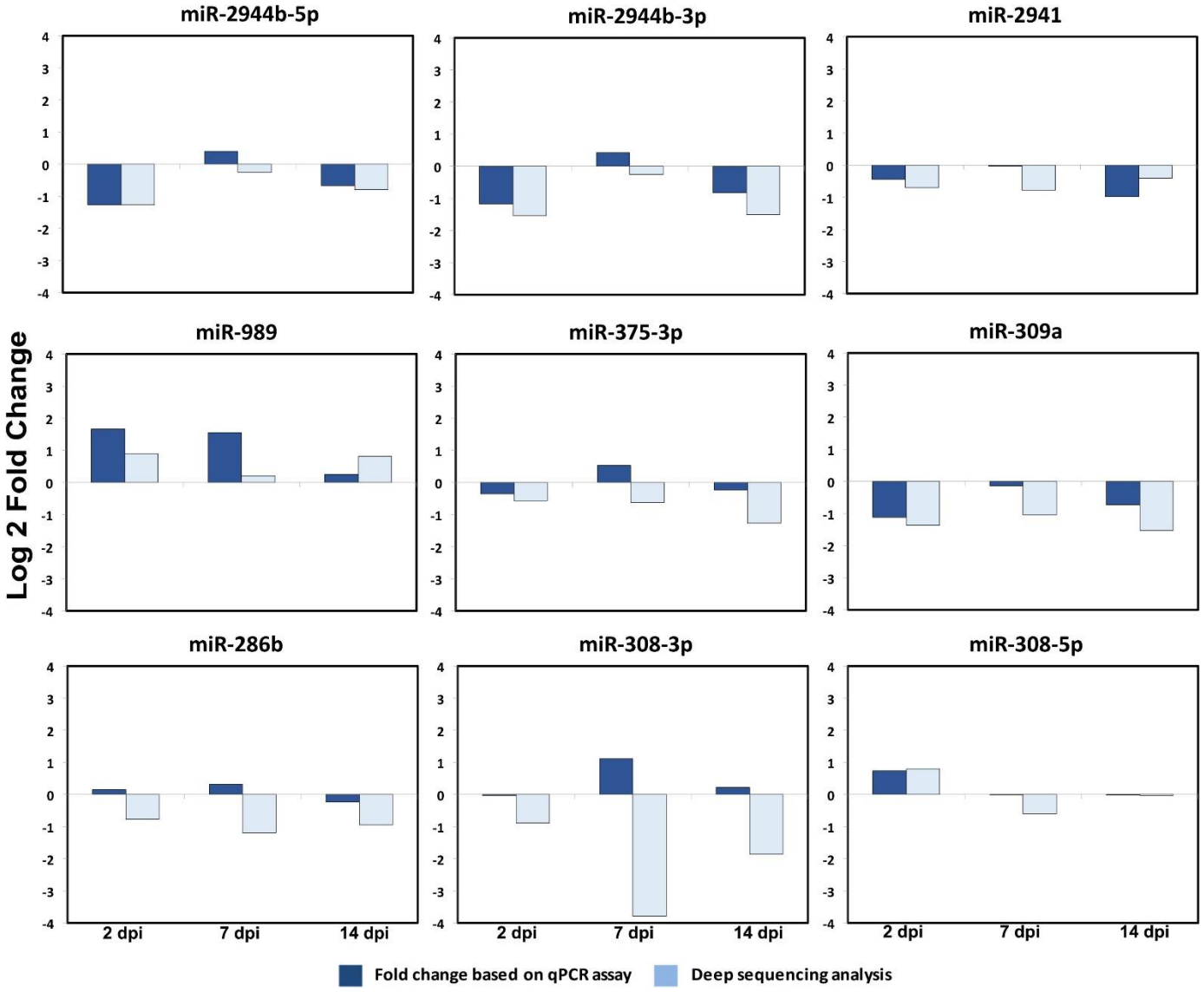

Fig 19. *Ae. aegypti* miRNAs are differentially expressed upon ZIKV infection.

The graphs show Log2 fold changes of a number of Ae. aegypti miRNAs based on deep sequencing data and RT-qPCR analysis of RNA samples from non-infected and ZIKV-infected mosquitoes at 2, 7 and 14 dpi. Fold changes are averages of three biological replicates.

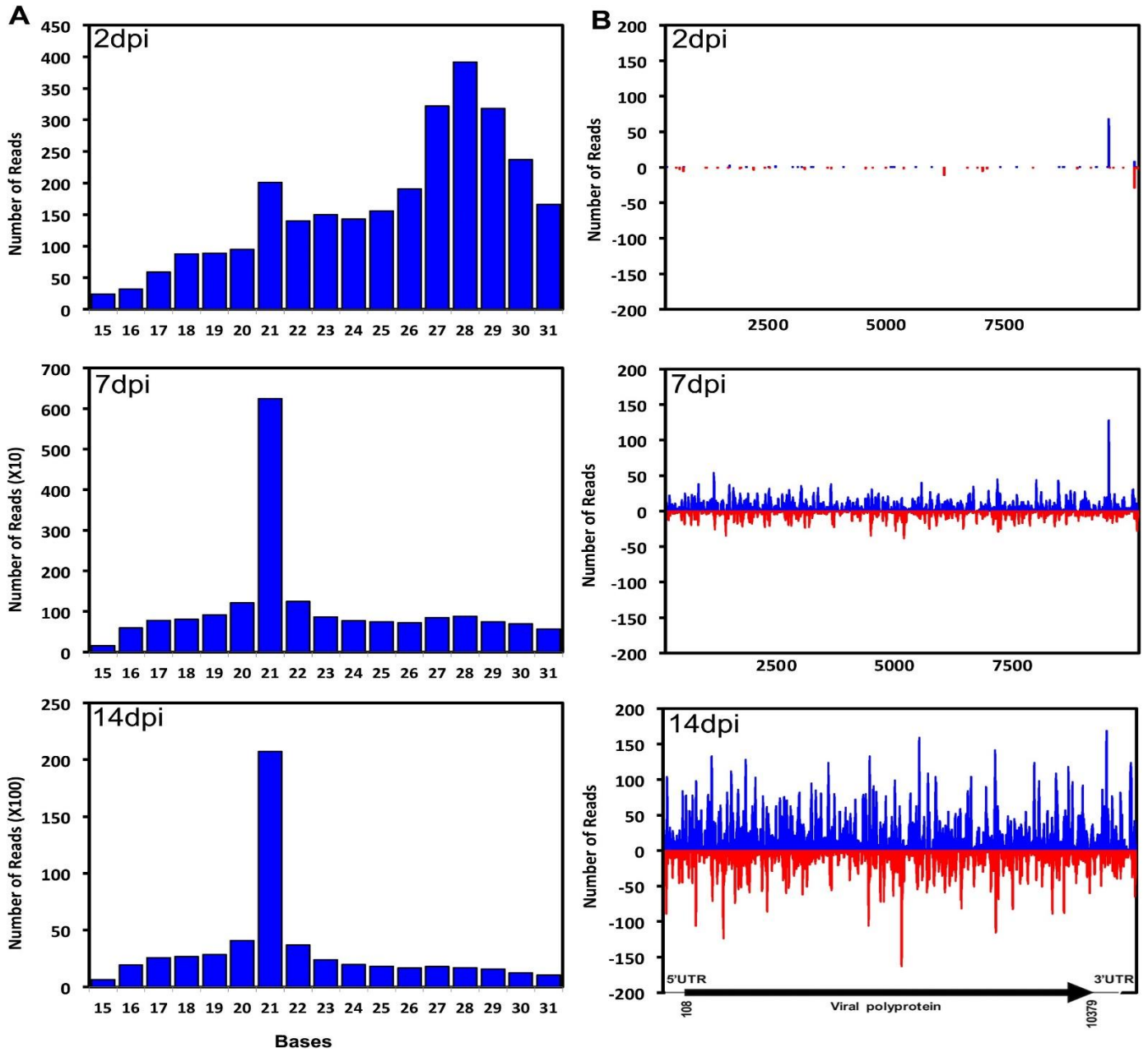

Fig 20. ZIKV elicits an RNAi response in *Ae. aegypti* mosquitoes.

(A) Length distribution of mappable reads to the ZIKV genome in small RNA libraries of Ae. aegypti mosquitoes at 2, 7 and 14 days post-inoculation (dpi). (B) Analysis of virus-derived short interfering RNAs (viRNAs) in Ae. aegypti ZIKV-infected mosquitoes. Distribution of 21 nt RNA reads that were mapped across the sense (blue) and anti-sense (red) strands of the ZIKV genome at 2, 7 and 14 dpi.

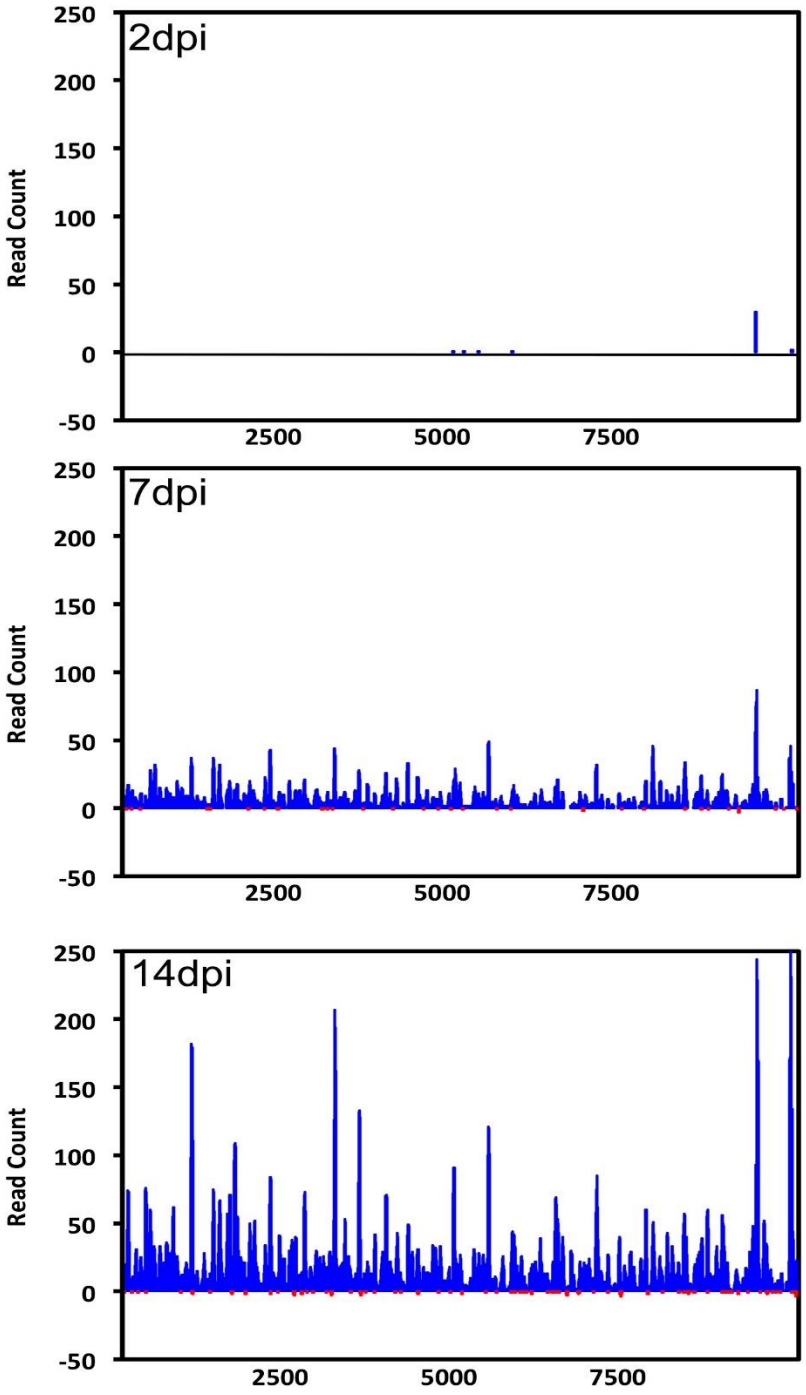

Fig 21. ZIKV-specific piRNA-like small RNAs in infected *Ae. aegypti* mosquitoes.

Distribution of 24–30 nt small RNAs that mapped across the sense (blue) and anti-sense (red) strands of the ZIKV genome at 2, 7 and 14 dpi.

Chapter 5 Summary

Ae. aegypti mosquitoes are medically important vectors for many human arboviruses including DENV, CHIKV, YFV and ZIKV. Many of the arboviruses spread by these mosquitoes have no treatment or prevention, forcing us to heavily rely on vector control to prevent their spread and transmission. However, recent and recurring global outbreaks of these viruses highlight the need for new vector control strategies to mitigate the spread of these vectors and more importantly the viruses they transmit. For this reason, the central hypothesis of this body of work was that mosquitoes, their microbiota and arboviruses, form an intimate tripartite relationship, in which each member influences one another either directly or indirectly. Therefore, this relationship can be utilized to develop novel self-sustaining microbial based vector control strategies that could be employed to reduce the burden of arboviral disease.

The first aim of this project explored the impact of different sugars on the mosquito microbiome using high throughput sequencing of the V3/4 of 16S rRNA gene and bioinformatics to analyze and compare the microbiome of mosquitoes from different sugar treatment groups, reared simultaneously in two different environments. In this study, we were able to demonstrate that the microbiome of different strains of mosquitoes have specific responses to different sugar types. More specifically, this work shows how different taxa of bacteria have different and preferential responses to each of these sugars and that these effects can have dramatic impacts on the composition of the microbiome diversity. Additionally, I examined the impact that using different sugars to modulate the microbiome might have on vector competence by infecting these mosquitoes via an

artificial blood meal containing 10^6 logs of ZIKV. Given that the mosquito background that I selected to test for vector competence yielded very subtle changes in response to different sugars, it was not surprising that sugar type had no impact on the vector competence of this line of mosquitoes. Future work testing vector competence in a mosquito line proven to have a microbiome susceptible to different sugars, like the New Orleans line reared in Liverpool, could potentially yield more interesting results about how the microbiome response to diet can impact mosquito infection. If this had resulted in changes in vector competence, it would provide a proof-of-principle that simply altering the carbohydrate source on which mosquitoes feed could be exploited as a vector control technique. Additional work examining how complex sugar blends or plants which have a composition of nectar that alters the microbiome of mosquitoes to induce refractoriness could be used in a vector control context.

The second aim focused on characterizing the relationship and interactions between the mosquito microbiome and ZIKV infection. For this project we explored the impact of the microbiome on ZIKV infection, as well as the impact of ZIKV infection on the mosquito microbiome. Modulating the microbiome through the use of tetracycline demonstrated that the bacterial families of *Acetobacteraceae* and *Enterobacteriaceae* are helping facilitate ZIKV infection in *Ae. aegypti.* Furthermore, I observed that the impact of antibiotic treatment on ZIKV infection in *Ae. aegypti* mosquitoes is independent of the immune response, further supporting a direct role of bacteria belonging to *Acetobacteraceae* and *Enterobacteriaceae* in increasing ZIKV susceptibility in mosquitoes. In field collected mosquitoes from Austin, TX., *Enterobacteriaceae* were

found to facilitate ZIKV infection, thus providing further evidence of their role in mosquito ZIKV infection. The lab strains of mosquitoes from the Rio Grande Valley (RGV) and Galveston, both demonstrated that ZIKV exposure impacted the total bacterial load of the microbiome and suggested that bacteria belonging to the family of *Acetobacteraceae* benefit from ZIKV exposure. This further validates the interaction between ZIKV and *Acetobacteraceae* that was observed in the antibiotic treatment experiment. Exploring the biological impact of mosquito isolates of *Asaia* and *Enterobacter* on ZIKV infection in *Ae. aegypti* resulted in virus strain specific responses. Here, we noted reduced infection and dissemination prevalence by *Asaia* compared to the sugar control group for Mex 1-7 strain of ZIKV, suggesting that this isolate could be making these mosquitoes refractory to ZIKV infection. *Cedecea* also exhibited a reduction in infection prevalence against this same strain of virus. The PRVABC-59 strain of ZIKV yielded different results, against this strain only *Cedecea* presented a decrease in viral titer against both the sugar control and *Asaia.* No differences were noted for dissemination titer levels or prevalence levels. All these findings taken together suggest that *Asaia* strains are likely contributing to ZIKV infection in mosquitoes while *Cedecea* strains could be impeding viral infection in mosquitoes and potentially are a candidate for microbial based vector control strategies. However, the idea of a single microbe that can completely inhibit ZIKV or any other arboviruses transmitted by mosquitoes likely does not exist in nature. Therefore, perhaps a more appropriate option for a microbial based vector control strategy may be to engineer a bacterium to produce dsRNA designed to block viruses. Nevertheless, before this can be carried out, the ideal

small RNA targets that could best utilize the RNAi pathway and target the virus must be identified.

The third and final aim for this work examines the relationship between *Ae. aegypti* mosquitoes and ZIKV infection. Small RNA's were sequenced, and the miRNA profile was examined between ZIKV infected and uninfected mosquitoes. Here, ZIKV was found to be inducing the RNAi response in mosquitoes through the production of virus derived short interfering RNAs. The RNAi response was found to have a bias for targeting the positive strand of the virus with many of the early targets producing high peaks around the NS5 region at the earlier timepoints during infection. Additionally, 17 host miRNAs were found to be impacted by ZIKV infection across three different time points, a number of which had also been linked to modulation by other arboviruses. Using these data we are now able to identify viRNA hot and cold-spots, thus allowing us to avoid decoy hot-spot targets and focus our efforts on the cold-spots which have been described as being more efficient at mediating a RNAi response to viral infection.

This work has examined the usefulness of sugar to modulate the microbiome and its potential impact on vector competence. Additionally, highlighting the role of specific bacterial taxa in enhancing ZIKV infection in mosquitoes and the intimate relationship between these bacteria and ZIKV. Finally, it also demonstrates the relationship between ZIKV infection and the mosquito host response, highlighting which areas of the viral genome are targeted by the host RNAi pathway at various time points post infection. These three aims serve as building blocks for the development of a microbial based

vector control strategy. Each aim emphasizes a specific relationship between mosquitoes, their microbiome and the viruses they transmit, which are the foundation of a tripartite interaction. When combined, the results from these aims begin to form the roadmap for the development of a robust microbial based vector control strategy aimed at reducing the spread of arboviruses by mosquitoes. These findings could be applied in an enhanced paratransgenesis (Wang *et al.*, 2012; Arora *et al.*, 2015; Wilke & Marrelli, 2015) stratagem utilizing microbiota in a type of mosquito immunization, in which modified bacteria could prevent ZIKV and other arboviral infections in these problematic vectors. In order to bring such a strategy to fruition, future studies must first identify specific bacterial isolates that have an innate inhibitory effect on arboviral infection in mosquitoes (Joyce *et al.*, 2011; Ramirez *et al.*, 2012, 2014). These isolates would then need to be engineered to deliver dsRNAs that can stimulate the RNAi pathway to target mosquito host genes that would induce a refractory phenotype coupled with important viral genome targets to significantly inhibit infection (Blair & Olson, 2015). Finally, the ideal bacterial candidate would also be vertically transferable across generations (Favia *et al.*, 2007; Damiani *et al.*, 2008), providing for a self-sustaining stratagem. The current climate of vector control opens the door for non-traditional approaches like microbial based strategies, therefore, the development of an enhanced paratransgenesis strategy would be a major contribution to fight against the spread of arboviral transmission.

Bibliography

Afanasiev, B.N., Ward, T.W., Beaty, B.J. & Carlson, J.O. (1999) Transduction ofAedes aegyptiMosquitoes with Vectors Derived fromAedesDensovirus. *Virology*, **257**, 62–72.

Aguiar, E.R.G.R., Olmo, R.P. & Marques, J.T. (2016) Virus-derived small RNAs: molecular footprints of host–pathogen interactions. *Wiley Interdisciplinary Reviews: RNA*, **7**, 824–837.

Ahluwalia, J.K., Khan, S.Z., Soni, K., Rawat, P., Gupta, A., Hariharan, M., *et al.* (2008) Human cellular microRNA hsa-miR-29a interferes with viral nef protein expression and HIV-1 replication. *Retrovirology*, **5**, 117.

Ahmed, I., Ehsan, M., Sin, Y., Paek, J., Khalid, N., Hayat, R., *et al.* (2014) Sphingobacterium pakistanensis sp. nov., a novel plant growth promoting rhizobacteria isolated from rhizosphere of Vigna mungo. *Antonie van Leeuwenhoek*, **105**, 325–333.

Akhoundi, M., Bakhtiari, R., Guillard, T., Baghaei, A., Tolouei, R., Sereno, D., *et al.* (2012) Diversity of the Bacterial and Fungal Microflora from the Midgut and Cuticle of Phlebotomine Sand Flies Collected in North-Western Iran. *PLoS ONE*, **7**, e50259.

Aliota, M.T., Peinado, S.A., Velez, I.D. & Osorio, J.E. (2016a) The wMel strain of Wolbachia Reduces Transmission of Zika virus by Aedes aegypti. *Scientific Reports*, **6**.

Aliota, M.T., Walker, E.C., Uribe Yepes, A., Dario Velez, I., Christensen, B.M. & Osorio, J.E. (2016b) The wMel Strain of Wolbachia Reduces Transmission of Chikungunya Virus in Aedes aegypti. *PLOS Neglected Tropical Diseases*, **10**, e0004677.

Al-Salem, W.S., Pigott, D.M., Subramaniam, K., Haines, L.R., Kelly-Hope, L., Molyneux, D.H., *et al.* (2016) Cutaneous Leishmaniasis and Conflict in Syria. *Emerging Infectious Diseases*, **22**, 931–933.

Althouse, B.M., Hanley, K.A., Diallo, M., Sall, A.A., Ba, Y., Faye, O., *et al.* (2015) Impact of Climate and Mosquito Vector Abundance on Sylvatic Arbovirus Circulation Dynamics in Senegal. *The American Journal of Tropical Medicine and Hygiene*, **92**, 88–97.

Anders, S. & Huber, W. (2010) Differential expression analysis for sequence count data. *Genome Biology*, **11**, R106.

Angleró-Rodríguez, Y.I., Blumberg, B.J., Dong, Y., Sandiford, S.L., Pike, A., Clayton, A.M., *et al.* (2016) A natural Anopheles-associated Penicillium chrysogenum enhances mosquito susceptibility to Plasmodium infection. *Scientific Reports*, **6**, 34084.

Angleró-Rodríguez, Y.I., MacLeod, H.J., Kang, S., Carlson, J.S., Jupatanakul, N. & Dimopoulos, G. (2017) Aedes aegypti Molecular Responses to Zika Virus: Modulation of Infection by the Toll and Jak/Stat Immune Pathways and Virus Host Factors. *Frontiers in Microbiology*, **8**.

Apte-Deshpande, A., Paingankar, M., Gokhale, M.D. & Deobagkar, D.N. (2012) Serratia odorifera a Midgut Inhabitant of Aedes aegypti Mosquito Enhances Its Susceptibility to Dengue-2 Virus. *PLoS ONE*, **7**, e40401.

Apte-Deshpande, A.D., Paingankar, M.S., Gokhale, M.D., Deobagkar, D.N. & others. (2014) Serratia odorifera mediated enhancement in susceptibility of Aedes aegypti for chikungunya virus. *The Indian journal of medical research*, **139**, 762.

Arora, A.K., Forshaw, A., Miller, T.A. & Durvasula, R. (2015) A delivery system for field application of paratransgenic control. *BMC Biotechnology*, **15**, 59.

Asgari, S. (2011) Role of MicroRNAs in Insect Host–Microorganism Interactions. *Frontiers in Physiology*, **2**.

Asgari, S. (2013) MicroRNA functions in insects. *Insect Biochemistry and Molecular Biology*, **43**, 388–397.

Asgari, S. (2014) Role of microRNAs in Arbovirus/Vector Interactions. *Viruses*, **6**, 3514–3534.

Azambuja, P., Feder, D. & Garcia, E.S. (2004) Isolation of Serratia marcescens in the midgut of Rhodnius prolixus: impact on the establishment of the parasite Trypanosoma cruzi in the vector. *Experimental Parasitology*, **107**, 89–96.

Bahia, A.C., Dong, Y., Blumberg, B.J., Mlambo, G., Tripathi, A., BenMarzouk-Hidalgo, O.J., *et al.* (2014) Exploring Anopheles gut bacteria for Plasmodium blocking activity. *Environmental Microbiology*, **16**, 2980–2994.

Baldini, F., Segata, N., Pompon, J., Marcenac, P., Robert Shaw, W., Dabiré, R.K., *et al.* (2014) Evidence of natural *Wolbachia* infections in field populations of *Anopheles gambiae*. *Nature Communications*, **5**, 3985.

Ballard, J.W.O. & Melvin, R.G. (2007) Tetracycline treatment influences mitochondrial metabolism and mtDNA density two generations after treatment in Drosophila. *Insect Molecular Biology*, **16**, 799–802.

Bando, H., Okado, K., Guelbeogo, W.M., Badolo, A., Aonuma, H., Nelson, B., *et al.* (2013) Intra-specific diversity of *Serratia marcescens* in *Anopheles* mosquito midgut defines *Plasmodium* transmission capacity. *Scientific Reports*, **3**, 1641.

Barletta, A.B.F., Alves, L.R., Nascimento Silva, M.C.L., Sim, S., Dimopoulos, G., Liechocki, S., *et al.* (2016) Emerging role of lipid droplets in Aedes aegypti immune response against bacteria and Dengue virus. *Scientific Reports*, **6**, 19928.

Barletta, A.B.F., Nascimento-Silva, M.C.L., Talyuli, O.A.C., Oliveira, J.H.M., Pereira, L.O.R., Oliveira, P.L., *et al.* (2017) Microbiota activates IMD pathway and limits Sindbis infection in Aedes aegypti. *Parasites & Vectors*, **10**.

Barreaux, A.M.G., Barreaux, P., Thievent, K. & Koella, J.C. (2016) Larval environment influences vector competence of the malaria mosquito, **7**, 6.

Barrett, A.D.T. (2016) Yellow Fever in Angola and Beyond — The Problem of Vaccine Supply and Demand. *New England Journal of Medicine*, **375**, 301–303.

Bassene, H., Niang, E.H.A., Fenollar, F., Dipankar, B., Doucouré, S., Ali, E., *et al.* (2018) 16S Metagenomic Comparison of Plasmodium falciparum–Infected and Noninfected Anopheles gambiae and Anopheles funestus Microbiota from Senegal. *The American Journal of Tropical Medicine and Hygiene*, **99**, 1489–1498.

Baton, L.A., Pacidônio, E.C., Gonçalves, D. da S. & Moreira, L.A. (2013) wFlu: Characterization and Evaluation of a Native Wolbachia from the Mosquito Aedes fluviatilis as a Potential Vector Control Agent. *PLOS ONE*, **8**, e59619.

Beard, C.B., Cordon-Rosales, C. & Durvasula, R.V. (2002) Bacterial Symbionts of the Triatominae and Their Potential Use in Control of Chagas Disease Transmission. *Annual Review of Entomology*, **47**, 123–141.

Behura, S.K., Gomez-Machorro, C., Harker, B.W., deBruyn, B., Lovin, D.D., Hemme, R.R., *et al.* (2011) Global Cross-Talk of Genes of the Mosquito Aedes aegypti in Response to Dengue Virus Infection. *PLoS Neglected Tropical Diseases*, **5**, e1385.

Beier, M.S., Pumpuni, C.B., Beier, J.C. & Davis, J.R. (1994) Effects of Para-Aminobenzoic Acid, Insulin, and Gentamicin on Plasmodium falciparum Development in Anopheline Mosquitoes (Diptera: Culicidae). *Journal of Medical Entomology*, **31**, 561–565.

Bennett, K.E., Olson, K.E., Muñoz, M. de L., Fernandez-Salas, I., Farfan-Ale, J.A., Higgs, S., *et al.* (2002) Variation in vector competence for dengue 2 virus among 24 collections of Aedes aegypti from Mexico and the United States. *The American Journal of Tropical Medicine and Hygiene*, **67**, 85–92.

Bern, C. (2015) Chagas' Disease. *New England Journal of Medicine*, **373**, 456–466.

Bhatt, S., Gething, P.W., Brady, O.J., Messina, J.P., Farlow, A.W., Moyes, C.L., *et al.* (2013) The global distribution and burden of dengue. *Nature*, **496**, 504–507.

Bian, G., Joshi, D., Dong, Y., Lu, P., Zhou, G., Pan, X., *et al.* (2013) Wolbachia Invades Anopheles stephensi Populations and Induces Refractoriness to Plasmodium Infection. *Science*, **340**, 748–751.

Bisi, D.C. & Lampe, D.J. (2011) Secretion of Anti-Plasmodium Effector Proteins from a Natural Pantoea agglomerans Isolate by Using PelB and HlyA Secretion Signals. *Appl. Environ. Microbiol.*, **77**, 4669–4675.

Blagrove, M.S.C., Arias-Goeta, C., Failloux, A.-B. & Sinkins, S.P. (2012) Wolbachia strain wMel induces cytoplasmic incompatibility and blocks dengue transmission in Aedes albopictus. *Proceedings of the National Academy of Sciences*, **109**, 255–260.

Blair, C.D. & Olson, K.E. (2015) The Role of RNA Interference (RNAi) in Arbovirus-Vector Interactions. *Viruses*, **7**, 820–843.

Blitvich, B.J. & Firth, A.E. (2015) Insect-Specific Flaviviruses: A Systematic Review of Their Discovery, Host Range, Mode of Transmission, Superinfection Exclusion Potential and Genomic Organization. *Viruses*, **7**, 1927–1959.

Boissière, A., Tchioffo, M.T., Bachar, D., Abate, L., Marie, A., Nsango, S.E., *et al.* (2012) Midgut Microbiota of the Malaria Mosquito Vector Anopheles gambiae and Interactions with Plasmodium falciparum Infection. *PLoS Pathogens*, **8**, e1002742.

Bolling, B.G., Olea-Popelka, F.J., Eisen, L., Moore, C.G. & Blair, C.D. (2012) Transmission dynamics of an insect-specific flavivirus in a naturally infected Culex pipiens laboratory colony and effects of co-infection on vector competence for West Nile virus. *Virology*, **427**, 90–97.

Bolling, B.G., Weaver, S.C., Tesh, R.B. & Vasilakis, N. (2015) Insect-Specific Virus Discovery: Significance for the Arbovirus Community. *Viruses*, **7**, 4911–4928.

Bongio, N.J. & Lampe, D.J. (2015) Inhibition of Plasmodium berghei Development in Mosquitoes by Effector Proteins Secreted from Asaia sp. Bacteria Using a Novel Native Secretion Signal. *PLOS ONE*, **10**, e0143541.

Boucias, D.G., Kariithi, H.M., Bourtzis, K., Schneider, D.I., Kelley, K., Miller, W.J., *et al.* (2013) Transgenerational Transmission of the Glossina pallidipes Hytrosavirus Depends on the Presence of a Functional Symbiome. *PLOS ONE*, **8**, e61150.

Bourtzis, K., Dobson, S.L., Xi, Z., Rasgon, J.L., Calvitti, M., Moreira, L.A., *et al.* (2014) Harnessing mosquito–Wolbachia symbiosis for vector and disease control. *Acta Tropica*, Biology and behaviour of male mosquitoes in relation to new approaches to control disease transmitting mosquitoes, **132**, S150–S163.

Brackney, D.E., Beane, J.E. & Ebel, G.D. (2009) RNAi targeting of West Nile virus in mosquito midguts promotes virus diversification. *PLoS Pathogens*, **5**, e1000502.

Brackney, D.E., Scott, J.C., Sagawa, F., Woodward, J.E., Miller, N.A., Schilkey, F.D., *et al.* (2010) C6/36 Aedes albopictus Cells Have a Dysfunctional Antiviral RNA Interference Response. *PLoS Neglected Tropical Diseases*, **4**, e856.

Brennecke, J., Aravin, A.A., Stark, A., Dus, M., Kellis, M., Sachidanandam, R., *et al.* (2007) Discrete Small RNA-Generating Loci as Master Regulators of Transposon Activity in Drosophila. *Cell*, **128**, 1089–1103.

Briones, A.M., Shililu, J., Githure, J., Novak, R. & Raskin, L. (2008) *Thorsellia anophelis* is the dominant bacterium in a Kenyan population of adult *Anopheles gambiae* mosquitoes. *The ISME Journal*, **2**, 74–82.

Broderick Nichole A. (2016) Friend, foe or food? Recognition and the role of antimicrobial peptides in gut immunity and Drosophila–microbe interactions. *Philosophical Transactions of the Royal Society B: Biological Sciences*, **371**, 20150295.

Buarque, D.S., Gomes, C.M., Araújo, R.N., Pereira, M.H., Ferreira, R.C., Guarneri, A.A., *et al.* (2016) A new antimicrobial protein from the anterior midgut of Triatoma infestans mediates Trypanosoma cruzi establishment by controlling the microbiota. *Biochimie*, **123**, 138–143.

Buchon, N., Broderick, N.A. & Lemaitre, B. (2013) Gut homeostasis in a microbial world: insights from *Drosophila melanogaster*. *Nature Reviews Microbiology*, **11**, 615–626.

Buck, M., Nilsson, L.K.J., Brunius, C., Dabiré, R.K., Hopkins, R. & Terenius, O. (2016) Bacterial associations reveal spatial population dynamics in Anopheles gambiae mosquitoes. *Scientific Reports*, **6**.

Bull, J.J. & Turelli, M. (2013) Wolbachia versus dengueEvolutionary forecasts. *Evolution, Medicine, and Public Health*, **2013**, 197–207.

Bustelo, X.R., Sauzeau, V. & Berenjeno, I.M. (2007) GTP-binding proteins of the Rho/Rac family: regulation, effectors and functions in vivo. *BioEssays*, **29**, 356–370.

Busto, G.U., Guven-Ozkan, T., Fulga, T.A., Vactor, D.V. & Davis, R.L. (2015) microRNAs That Promote or Inhibit Memory Formation in Drosophila melanogaster. *Genetics*, **200**, 569–580.

Calvitti, M., Marini, F., Desiderio, A., Puggioli, A. & Moretti, R. (2015) Wolbachia density and cytoplasmic incompatibility in Aedes albopictus: concerns with using artificial Wolbachia infection as a vector suppression tool. *PloS One*, **10**, e0121813.

Campbell, C.L., Harrison, T., Hess, A.M. & Ebel, G.D. (2014) MicroRNA levels are modulated in Aedes aegypti after exposure to Dengue-2. *Insect Molecular Biology*, **23**, 132–139.

Caragata, E.P., Rancès, E., Hedges, L.M., Gofton, A.W., Johnson, K.N., O'Neill, S.L., *et al.* (2013) Dietary Cholesterol Modulates Pathogen Blocking by Wolbachia. *PLOS Pathogens*, **9**, e1003459.

Carissimo, G., Pondeville, E., McFarlane, M., Dietrich, I., Mitri, C., Bischoff, E., *et al.* (2015) Antiviral immunity of Anopheles gambiae is highly compartmentalized, with distinct roles for RNA interference and gut microbiota. *Proceedings of the National Academy of Sciences*, **112**, E176–E185.

Casadevall, A., Fang, F.C. & Pirofski, L. (2011) Microbial Virulence as an Emergent Property: Consequences and Opportunities. *PLOS Pathogens*, **7**, e1002136.

Castillo, J.A., Castrillón, J.C., Diosa-Toro, M., Betancur, J.G., St Laurent, G., Smit, J.M., *et al.* (2016) Complex interaction between dengue virus replication and expression of miRNA-133a. *BMC Infectious Diseases*, **16**, 29.

Castro, D.P., Moraes, C.S., Garcia, E.S. & Azambuja, P. (2007) Inhibitory effects of d-mannose on trypanosomatid lysis induced by Serratia marcescens. *Experimental Parasitology*, **115**, 200–204.

Castro, D.P., Moraes, C.S., Gonzalez, M.S., Ratcliffe, N.A., Azambuja, P. & Garcia, E.S. (2012) Trypanosoma cruzi Immune Response Modulation Decreases Microbiota in Rhodnius prolixus Gut and Is Crucial for Parasite Survival and Development. *PLOS ONE*, **7**, e36591.

Chalcoff, V.R., Aizen, M.A. & Galetto, L. (2006) Nectar Concentration and Composition of 26 Species from the Temperate Forest of South America. *Annals of Botany*, **97**, 413–421.

Chandler, J.A., Liu, R.M. & Bennett, S.N. (2015) RNA shotgun metagenomic sequencing of northern California (USA) mosquitoes uncovers viruses, bacteria, and fungi. *Frontiers in Microbiology*, **6**.

Chandler, J.A., Morgan Lang, J., Bhatnagar, S., Eisen, J.A. & Kopp, A. (2011) Bacterial Communities of Diverse Drosophila Species: Ecological Context of a Host–Microbe Model System. *PLoS Genetics*, **7**, e1002272.

Chavshin, A.R., Oshaghi, M.A., Vatandoost, H., Yakhchali, B., Zarenejad, F. & Terenius, O. (2015) Malpighian tubules are important determinants of Pseudomonas transstadial transmission and longtime persistence in Anopheles stephensi. *Parasites & vectors*, **8**.

Chen, S., Bagdasarian, M. & Walker, E.D. (2015a) Elizabethkingia anophelis: Molecular Manipulation and Interactions with Mosquito Hosts. *Appl. Environ. Microbiol.*, **81**, 2233–2243.

Chen, X., Li, L., Hu, Q., Zhang, B., Wu, W., Jin, F., *et al.* (2015b) Expression of dsRNA in recombinant Isaria fumosorosea strain targets the TLR7 gene in Bemisia tabaci. *BMC Biotechnology*, **15**.

Chen, Z. & Kearney, C.M. (2015) Nectar protein content and attractiveness to Aedes aegypti and Culex pipiens in plants with nectar/insect associations. *Acta Tropica*, **146**, 81–88.

Chouaia, B., Gaiarsa, S., Crotti, E., Comandatore, F., Degli Esposti, M., Ricci, I., *et al.* (2014) Acetic Acid Bacteria Genomes Reveal Functional Traits for Adaptation to Life in Insect Guts. *Genome Biology and Evolution*, **6**, 912–920.

Chouaia, B., Rossi, P., Epis, S., Mosca, M., Ricci, I., Damiani, C., *et al.* (2012) Delayed larval development in Anopheles mosquitoes deprived of Asaiabacterial symbionts. *BMC Microbiology*, **12**, S2.

Ciota, A.T., Bialosuknia, S.M., Zink, S.D., Brecher, M., Ehrbar, D.J., Morrissette, M.N., *et al.* (2017) Effects of Zika Virus Strain and *Aedes* Mosquito Species on Vector Competence. *Emerging Infectious Diseases*, **23**, 1110–1117.

Cirimotich, C.M., Dong, Y., Clayton, A.M., Sandiford, S.L., Souza-Neto, J.A., Mulenga, M., *et al.* (2011) Natural microbe-mediated refractoriness to Plasmodium infection in Anopheles gambiae. *Science (New York, NY)*, **332**, 855–858.

Clayton, K.A., Gall, C.A., Mason, K.L., Scoles, G.A. & Brayton, K.A. (2015) The characterization and manipulation of the bacterial microbiome of the Rocky Mountain wood tick, Dermacentor andersoni. *Parasites & Vectors*, **8**, 632.

Coon, K.L., Brown, M.R. & Strand, M.R. (2016) Mosquitoes host communities of bacteria that are essential for development but vary greatly between local habitats. *Molecular Ecology*, **25**, 5806–5826.

Coon, K.L., Vogel, K.J., Brown, M.R. & Strand, M.R. (2014) Mosquitoes rely on their gut microbiota for development. *Molecular Ecology*, **23**, 2727–2739.

Cox, J., Brown, H.E. & Rico-Hesse, R. (2011) Variation in Vector Competence for Dengue Viruses Does Not Depend on Mosquito Midgut Binding Affinity. *PLoS Neglected Tropical Diseases*, **5**, e1172.

Crockett, R.K., Burkhalter, K., Mead, D., Kelly, R., Brown, J., Varnado, W., *et al.* (2012) Culex Flavivirus and West Nile Virus in Culex quinquefasciatus Populations in the Southeastern United States. *Journal of Medical Entomology*, **49**, 165–174.

Crotti, E., Rizzi, A., Chouaia, B., Ricci, I., Favia, G., Alma, A., *et al.* (2010) Acetic Acid Bacteria, Newly Emerging Symbionts of Insects. *Applied and Environmental Microbiology*, **76**, 6963–6970.

Damiani, C., Ricci, I., Crotti, E., Rossi, P., Rizzi, A., Scuppa, P., *et al.* (2008) Paternal transmission of symbiotic bacteria in malaria vectors. *Current Biology*, **18**, R1087–R1088.

Damiani, C., Ricci, I., Crotti, E., Rossi, P., Rizzi, A., Scuppa, P., *et al.* (2010) Mosquito-Bacteria Symbiosis: The Case of Anopheles gambiae and Asaia. *Microbial Ecology*.

Daneshvar, K., Nath, S., Khan, A., Shover, W., Richardson, C. & Goodliffe, J.M. (2013) MicroRNA miR-308 regulates dMyc through a negative feedback loop in Drosophila. *Biology Open*, **2**, 1–9.

Dantas-Torres, F., Chomel, B.B. & Otranto, D. (2012) Ticks and tick-borne diseases: a One Health perspective. *Trends in Parasitology*, **28**, 437–446.

David, M.R., Santos, L.M.B. dos, Vicente, A.C.P. & Maciel-de-Freitas, R. (2016) Effects of environment, dietary regime and ageing on the dengue vector microbiota: evidence of a core microbiota throughout Aedes aegypti lifespan. *Memórias do Instituto Oswaldo Cruz*, 0–0.

De Vooght, L., Caljon, G., De Ridder, K. & Van Den Abbeele, J. (2014) Delivery of a functional anti-trypanosome Nanobody in different tsetse fly tissues via a bacterial symbiont, Sodalis glossinidius. *Microbial Cell Factories*, **13**, 156.

De Vooght, L., Caljon, G., Stijlemans, B., De Baetselier, P., Coosemans, M. & Van Den Abbeele, J. (2012) Expression and extracellular release of a functional anti-trypanosome Nanobody® in Sodalis glossinidius, a bacterial symbiont of the tsetse fly. *Microbial Cell Factories*, **11**, 23.

Dennison, N.J., Jupatanakul, N. & Dimopoulos, G. (2014) The mosquito microbiota influences vector competence for human pathogens. *Current Opinion in Insect Science*, **3**, 6–13.

Diallo, D., Sall, A.A., Diagne, C.T., Faye, O., Faye, O., Ba, Y., *et al.* (2014) Zika Virus Emergence in Mosquitoes in Southeastern Senegal, 2011. *PLOS ONE*, **9**, e109442.

Dick, G.W.A., Kitchen, S.F. & Haddow, A.J. (1952) Zika Virus (I). Isolations and serological specificity. *Transactions of The Royal Society of Tropical Medicine and Hygiene*, **46**, 509–520.

Dickson, L.B., Ghozlane, A., Volant, S., Bouchier, C., Ma, L., Vega-Rua, A., *et al.* (2017a) Diverse laboratory colonies of Aedes aegypti harbor the same adult midgut bacterial microbiome.

Dickson, L.B., Jiolle, D., Minard, G., Moltini-Conclois, I., Volant, S., Ghozlane, A., *et al.* (2017b) Carryover effects of larval exposure to different environmental bacteria drive adult trait variation in a mosquito vector. *Science Advances*, **3**, e1700585.

Dietrich, I., Jansen, S., Fall, G., Lorenzen, S., Rudolf, M., Huber, K., *et al.* (2017) RNA Interference Restricts Rift Valley Fever Virus in Multiple Insect Systems. *mSphere*, **2**, e00090-17.

Dimopoulos, G., Richman, A., Muller, H.-M. & Kafatos, F.C. (1997) Molecular immune responses of the mosquito Anopheles gambiae to bacteria and malaria parasites. *Proceedings of the National Academy of Sciences*, **94**, 11508–11513.

Dong, Y., Manfredini, F. & Dimopoulos, G. (2009) Implication of the Mosquito Midgut Microbiota in the Defense against Malaria Parasites. *PLoS Pathogens*, **5**, e1000423.

Dong, Y., Morton, J.C., Ramirez, J.L., Souza-Neto, J.A. & Dimopoulos, G. (2012) The entomopathogenic fungus Beauveria bassiana activate toll and JAK-STAT pathway-controlled effector genes and anti-dengue activity in Aedes aegypti. *Insect Biochemistry and Molecular Biology*, **42**, 126–132.

Duguma, D., Hall, M.W., Rugman-Jones, P., Stouthamer, R., Terenius, O., Neufeld, J.D., *et al.* (2015) Developmental succession of the microbiome of Culex mosquitoes. *BMC Microbiology*, **15**.

Duguma, D., Hall, M.W., Smartt, C.T., Debboun, M. & Neufeld, J.D. (2019) Microbiota variations in *Culex nigripalpus* disease vector mosquito of West Nile virus and Saint Louis Encephalitis from different geographic origins. *PeerJ*, **6**, e6168.

Duron, O., Bouchon, D., Boutin, S., Bellamy, L., Zhou, L., Engelstädter, J., *et al.* (2008) The diversity of reproductive parasites among arthropods: Wolbachiado not walk alone. *BMC Biology*, **6**, 27.

Durvasula, R.V., Gumbs, A., Panackal, A., Kruglov, O., Aksoy, S., Merrifield, R.B., *et al.* (1997) Prevention of insect-borne disease: An approach using transgenic symbiotic bacteria. *Proceedings of the National Academy of Sciences*, **94**, 3274–3278.

Durvasula, R.V., Kroger, A., Goodwin, M., Panackal, A., Kruglov, O., Taneja, J., *et al.* (1999) Strategy for Introduction of Foreign Genes into Field Populations of Chagas Disease Vectors. *Annals of the Entomological Society of America*, **92**, 937–943.

Dutra, H.L.C., Caragata, E.P. & Moreira, L.A. (2016) The re-emerging arboviral threat: Hidden enemies: The emergence of obscure arboviral diseases, and the potential use of *Wolbachia* in their control. *BioEssays*, 1600175.

Ebel, G.D., Rochlin, I., Longacker, J. & Kramer, L.D. (2005) Culex restuans (Diptera: Culicidae) Relative Abundance and Vector Competence for West Nile Virus. *JOURNAL OF MEDICAL ENTOMOLOGY*, **42**, 6.

Edman, J.D., Kittayapong, P., Day, J.F., Naksathit, A. & Scott, T.W. (1997) A Fitness Advantage for Aedes aegypti and the Viruses It Transmits When Females Feed Only on Human Blood. *The American Journal of Tropical Medicine and Hygiene*, **57**, 235–239.

Edman, J.D., Strickman, D., Kittayapong, P. & Scott, T.W. (1992) Female Aedes aegypti (Diptera: Culicidae) in Thailand rarely feed on sugar. *Journal of Medical Entomology*, **29**, 1035–1038.

Eichler, S. & Schaub, G.A. (2002) Development of Symbionts in Triatomine Bugs and the Effects of Infections with Trypanosomatids. *Experimental Parasitology*, **100**, 17–27.

Engel, P., Martinson, V.G. & Moran, N.A. (2012) Functional diversity within the simple gut microbiota of the honey bee. *Proceedings of the National Academy of Sciences*, **109**, 11002–11007.

Engel, P. & Moran, N.A. (2013) The gut microbiota of insects – diversity in structure and function. *FEMS Microbiology Reviews*, **37**, 699–735.

Engel, P., Stepanauskas, R. & Moran, N.A. (2014) Hidden Diversity in Honey Bee Gut Symbionts Detected by Single-Cell Genomics. *PLoS Genetics*, **10**, e1004596.

Enright, A.J., John, B., Gaul, U., Tuschl, T., Sander, C. & Marks, D.S. (2003) MicroRNA targets in Drosophila. *Genome Biology*, **5**, R1.

Erkosar, B., Storelli, G., Defaye, A. & Leulier, F. (2013) Host-Intestinal Microbiota Mutualism: "Learning on the Fly." *Cell Host & Microbe*, **13**, 8–14.

Fang, W., Vega-Rodríguez, J., Ghosh, A.K., Jacobs-Lorena, M., Kang, A. & Leger, R.J.S. (2011) Development of Transgenic Fungi That Kill Human Malaria Parasites in Mosquitoes. *Science*, **331**, 1074–1077.

Favia, G., Ricci, I., Damiani, C., Raddadi, N., Crotti, E., Marzorati, M., *et al.* (2007) Bacteria of the genus Asaia stably associate with Anopheles stephensi, an Asian malarial mosquito vector. *Proceedings of the National Academy of Sciences*, **104**, 9047–9051.

Figueira-Mansur, J., Ferreira-Pereira, A., Mansur, J.F., Franco, T.A., Alvarenga, E.S.L., Sorgine, M.H.F., *et al.* (2013) Silencing of P-glycoprotein increases mortality in temephos-treated Aedes aegypti larvae. *Insect Molecular Biology*, **22**, 648–658.

Foronda, D., Weng, R., Verma, P., Chen, Y.-W. & Cohen, S.M. (2014) Coordination of insulin and Notch pathway activities by microRNA miR-305 mediates adaptive homeostasis in the intestinal stem cells of the Drosophila gut. *Genes & Development*, **28**, 2421–2431.

Foster, W.A. (1995) Mosquito sugar feeding and reproductive energetics. *Annual review of entomology*, **40**, 443–474.

Frank, T., Gody, J.C., Nguyen, L.B.L., Berthet, N., Fleche-Mateos, A.L., Bata, P., *et al.* (2013) First case of Elizabethkingia anophelis meningitis in the Central African Republic. *The Lancet*, **381**, 1876.

Fryxell, R.T.T. & DeBruyn, J.M. (2016) The Microbiome of Ehrlichia-Infected and Uninfected Lone Star Ticks (Amblyomma americanum). *PLOS ONE*, **11**, e0146651.

Gall, C.A., Reif, K.E., Scoles, G.A., Mason, K.L., Mousel, M., Noh, S.M., *et al.* (2016) The bacterial microbiome of *Dermacentor andersoni* ticks influences pathogen susceptibility. *The ISME Journal*, **10**, 1846–1855.

Garay, J.A.R., Hughes, G.L., Koundal, V., Rasgon, J.L. & Mwangi, M.M. (2016) Genome Sequence of Elizabethkingia anophelis Strain EaAs1, Isolated from the Asian Malaria Mosquito Anopheles stephensi. *Genome Announc.*, **4**, e00084-16.

Garver, L.S., Dong, Y. & Dimopoulos, G. (2009) Caspar Controls Resistance to Plasmodium falciparum in Diverse Anopheline Species. *PLoS Pathogens*, **5**, e1000335.

Genes, C., Baquero, E., Echeverri, F., Maya, J.D. & Triana, O. (2011) Mitochondrial dysfunction in Trypanosoma cruzi: the role of Serratia marcescens prodigiosin in the alternative treatment of Chagas disease. *Parasites & vectors*, **4**, 1.

Gimonneau, G., Tchioffo, M.T., Abate, L., Boissière, A., Awono-Ambéné, P.H., Nsango, S.E., *et al.* (2014) Composition of Anopheles coluzzii and Anopheles gambiae microbiota from larval to adult stages. *Infection, Genetics and Evolution*, **28**, 715–724.

Glaser, R.L. & Meola, M.A. (2010) The Native Wolbachia Endosymbionts of Drosophila melanogaster and Culex quinquefasciatus Increase Host Resistance to West Nile Virus Infection. *PLOS ONE*, **5**, e11977.

Göertz, G.P., Fros, J.J., Miesen, P., Vogels, C.B.F., Bent, M.L. van der, Geertsema, C., *et al.* (2016) Noncoding Subgenomic Flavivirus RNA Is Processed by the Mosquito RNA Interference Machinery and Determines West Nile Virus Transmission by Culex pipiens Mosquitoes. *Journal of Virology*, **90**, 10145–10159.

Gonçalves, C.M., Melo, F.F., Bezerra, J.M., Chaves, B.A., Silva, B.M., Silva, L.D., *et al.* (2014) Distinct variation in vector competence among nine field populations of Aedes aegypti from a Brazilian dengue-endemic risk city. *Parasites & Vectors*, **7**, 320.

Gopinath, S., Kim, M.V., Rakib, T., Wong, P.W., Zandt, M. van, Barry, N.A., *et al.* (2018) Topical application of aminoglycoside antibiotics enhances host resistance to viral infections in a microbiota-independent manner. *Nature Microbiology*, **3**, 611.

Gorman, M.J. & Paskewitz, S.M. (2000) Persistence of Infection in Mosquitoes Injected with Bacteria. *Journal of Invertebrate Pathology*, **75**, 296–297.

Gu, J.-B., Dong, Y.-Q., Peng, H.-J. & Chen, X.-G. (2010) A Recombinant AeDNA Containing the Insect-Specific Toxin, BmK IT1, Displayed an Increasing Pathogenicity on Aedes albopictus. *The American Journal of Tropical Medicine and Hygiene*, **83**, 614–623.

Gu, W., Müller, G., Schlein, Y., Novak, R.J. & Beier, J.C. (2011) Natural Plant Sugar Sources of Anopheles Mosquitoes Strongly Impact Malaria Transmission Potential. *PLOS ONE*, **6**, e15996.

Gubler, D.J. & Rosen, L. (1976) Variation among geographic strains of Aedes albopictus in susceptibility to infection with dengue viruses. *The American Journal of Tropical Medicine and Hygiene*, **25**, 318–325.

Guégan, M., Minard, G., Tran, F.-H., Tran Van, V., Dubost, A. & Valiente Moro, C. (2018) Short-term impacts of anthropogenic stressors on Aedes albopictus mosquito vector microbiota. *FEMS Microbiology Ecology*, **94**.

Gulia-Nuss, M., Eum, J.-H., Strand, M.R. & Brown, M.R. (2012) Ovary ecdysteroidogenic hormone activates egg maturation in the mosquito Georgecraigius atropalpus after adult eclosion or a blood meal. *The Journal of Experimental Biology*, **215**, 3758–3767.

Gusmão, D.S., Santos, A.V., Marini, D.C., Bacci, M., Berbert-Molina, M.A. & Lemos, F.J.A. (2010) Culture-dependent and culture-independent characterization of microorganisms associated with Aedes aegypti (Diptera: Culicidae) (L.) and dynamics of bacterial colonization in the midgut. *Acta Tropica*, **115**, 275–281.

Hall-Mendelin, S., McLean, B.J., Bielefeldt-Ohmann, H., Hobson-Peters, J., Hall, R.A. & Hurk, A.F. van den. (2016) The insect-specific Palm Creek virus modulates West Nile virus infection in and transmission by Australian mosquitoes. *Parasites & Vectors*, **9**, 414.

Harrington, L.C., Fleisher, A., Ruiz-Moreno, D., Vermeylen, F., Wa, C.V., Poulson, R.L., *et al.* (2014) Heterogeneous Feeding Patterns of the Dengue Vector, Aedes aegypti, on Individual Human Hosts in Rural Thailand. *PLoS Neglected Tropical Diseases*, **8**, e3048.

Hawley, W.A., Pumpuni, C.B., Brady, R.H. & Craig, G.B. (1989) Overwintering Survival of Aedes albopictus (Diptera: Culicidae) Eggs in Indiana. *Journal of Medical Entomology*, **26**, 122–129.

He, M., Zhang, H., Li, Y., Wang, G., Tang, B., Zhao, J., *et al.* (2018) Cathelicidin-Derived Antimicrobial Peptides Inhibit Zika Virus Through Direct Inactivation and Interferon Pathway. *Frontiers in Immunology*, **9**.

Hedges, L.M., Brownlie, J.C., O'Neill, S.L. & Johnson, K.N. (2008) Wolbachia and Virus Protection in Insects. *Science*, **322**, 702–702.

Hegde, S., Khanipov, K., Albayrak, L., Golovko, G., Pimenova, M., Saldaña, M.A., *et al.* (2018) Microbiome Interaction Networks and Community Structure From Laboratory-Reared and Field-Collected Aedes aegypti, Aedes albopictus, and Culex quinquefasciatus Mosquito Vectors. *Frontiers in Microbiology*, **9**.

Hegde, S., Rasgon, J.L. & Hughes, G.L. (2015) The microbiome modulates arbovirus transmission in mosquitoes. *Current Opinion in Virology*, **15**, 97–102.

Hess, A.M., Prasad, A.N., Ptitsyn, A., Ebel, G.D., Olson, K.E., Barbacioru, C., *et al.* (2011) Small RNA profiling of Dengue virus-mosquito interactions implicates the PIWI RNA pathway in anti-viral defense. *BMC microbiology*, **11**, 1.

Hibbing, M.E., Fuqua, C., Parsek, M.R. & Peterson, S.B. (2010) Bacterial competition: surviving and thriving in the microbial jungle. *Nature Reviews Microbiology*, **8**, 15–25.

Hilgenboecker, K., Hammerstein, P., Schlattmann, P., Telschow, A. & Werren, J.H. (2008) How many species are infected with Wolbachia? – a statistical analysis of current data. *FEMS Microbiology Letters*, **281**, 215–220.

Hill, C.L., Sharma, A., Shouche, Y. & Severson, D.W. (2014) Dynamics of midgut microflora and dengue virus impact on life history traits in Aedes aegypti. *Acta Tropica*, **140**, 151–157.

Hillesland, H., Read, A., Subhadra, B., Hurwitz, I., McKelvey, R., Ghosh, K., *et al.* (2008) Identification of aerobic gut bacteria from the kala azar vector, Phlebotomus argentipes: a platform for potential paratransgenic manipulation of sand flies. *The American journal of tropical medicine and hygiene*, **79**, 881–886.

Hoffmann, A.A., Iturbe-Ormaetxe, I., Callahan, A.G., Phillips, B.L., Billington, K., Axford, J.K., *et al.* (2014) Stability of the wMel Wolbachia Infection following Invasion into Aedes aegypti Populations. *PLOS Neglected Tropical Diseases*, **8**, e3115.

Hoffmann, A.A., Montgomery, B.L., Popovici, J., Iturbe-Ormaetxe, I., Johnson, P.H., Muzzi, F., *et al.* (2011) Successful establishment of *Wolbachia* in *Aedes* populations to suppress dengue transmission. *Nature*, **476**, 454–457.

Huang, X.-F., Chaparro, J.M., Reardon, K.F., Judd, T.M. & Vivanco, J.M. (2016) Supplementing Blends of Sugars, Amino Acids, and Secondary Metabolites to the Diet of Termites (Reticulitermes flavipes) Drive Distinct Gut Bacterial Communities. *Microbial Ecology*, **72**, 497–502.

Hughes, G.L., Allsopp, P.G., Webb, R.I., Yamada, R., Iturbe-Ormaetxe, I., Brumbley, S.M., *et al.* (2011a) Identification of Yeast Associated with the Planthopper, Perkinsiella saccharicida: Potential Applications for Fiji Leaf Gall Control. *Current Microbiology*, **63**, 392–401.

Hughes, G.L., Dodson, B.L., Johnson, R.M., Murdock, C.C., Tsujimoto, H., Suzuki, Y., *et al.* (2014a) Native microbiome impedes vertical transmission of Wolbachia in Anopheles mosquitoes. *Proceedings of the National Academy of Sciences*, **111**, 12498 – 12503.

Hughes, G.L., Koga, R., Xue, P., Fukatsu, T. & Rasgon, J.L. (2011b) Wolbachia Infections Are Virulent and Inhibit the Human Malaria Parasite Plasmodium Falciparum in Anopheles Gambiae. *PLOS Pathogens*, **7**, e1002043.

Hughes, G.L. & Rasgon, J.L. (2014) Transinfection: a method to investigate Wolbachia–host interactions and control arthropod-borne disease. *Insect Molecular Biology*, **23**, 141–151.

Hughes, G.L., Rivero, A. & Rasgon, J.L. (2014b) Wolbachia Can Enhance Plasmodium Infection in Mosquitoes: Implications for Malaria Control? *PLoS Pathogens*, **10**, e1004182.

Hughes, G.L., Vega-Rodriguez, J., Xue, P. & Rasgon, J.L. (2012) Wolbachia Strain wAlbB Enhances Infection by the Rodent Malaria Parasite Plasmodium berghei in Anopheles gambiae Mosquitoes. *Appl. Environ. Microbiol.*, **78**, 1491–1495.

Hurk, A.F. van den, Hall-Mendelin, S., Pyke, A.T., Frentiu, F.D., McElroy, K., Day, A., *et al.* (2012) Impact of Wolbachia on Infection with Chikungunya and Yellow Fever Viruses in the Mosquito Vector Aedes aegypti. *PLOS Neglected Tropical Diseases*, **6**, e1892.

Hussain, M., Etebari, K. & Asgari, S. (2016) Chapter Seven - Functions of Small RNAs in Mosquitoes. In *Advances in Insect Physiology*, Progress in Mosquito Research (ed. by Raikhel, A.S.). Academic Press, pp. 189–222.

Hussain, M., Walker, T., O'Neill, S.L. & Asgari, S. (2013) Blood meal induced microRNA regulates development and immune associated genes in the Dengue mosquito vector, Aedes aegypti. *Insect Biochemistry and Molecular Biology*, **43**, 146–152.

Huys, G., Coopman, V., Van Varenbergh, D. & Cordonnier, J. (2012) Selective culturing and genus-specific PCR detection for identification of Aeromonas in tissue samples to assist the medico-legal diagnosis of death by drowning. *Forensic Science International*, **221**, 11–15.

Ignatova, E.A., Nagornaia, S.S., Povazhnaia, T.N. & Ianishevskaia, G.S. (1996) [The yeast flora of blood-sucking mosquitoes]. *Mikrobiolohichnyi zhurnal (Kiev, Ukraine : 1993)*, **58**, 12–15.

Imam, H., Sofi, G., Zarnigar & Aziz, S. (2014) The basic rules and methods of mosquito rearing (Aedes aegypti). *Tropical Parasitology*, **4**, 53.

Isoe, J., Collins, J., Badgandi, H., Day, W.A. & Miesfeld, R.L. (2011) Defects in coatomer protein I (COPI) transport cause blood feeding-induced mortality in Yellow Fever mosquitoes. *Proceedings of the National Academy of Sciences*, **108**, E211–E217.

Jasinskas, A., Zhong, J. & Barbour, A.G. (2007) Highly Prevalent Coxiella sp. Bacterium in the Tick Vector Amblyomma americanum. *Appl. Environ. Microbiol.*, **73**, 334–336.

Jeffries, C.L. & Walker, T. (2015) The Potential Use of Wolbachia-Based Mosquito Biocontrol Strategies for Japanese Encephalitis. *PLOS Neglected Tropical Diseases*, **9**, e0003576.

Jeffries, C.L. & Walker, T. (2016) Wolbachia Biocontrol Strategies for Arboviral Diseases and the Potential Influence of Resident Wolbachia Strains in Mosquitoes. *Current Tropical Medicine Reports*, **3**, 20–25.

Johnson, H.A. (1947) Practical laboratory methods for quantity rearing and handling of Aedes aegypti mosquitoes to be infected with Plasmodium gallinaceum. *Journal of the National Malaria Society*, **6**, 173–179.

Joshi, D., McFadden, M.J., Bevins, D., Zhang, F. & Xi, Z. (2014) Wolbachia strain w AlbB confers both fitness costs and benefit on Anopheles stephensi. *Parasites & Vectors*, **7**, 336.

Joubert, D.A., Walker, T., Carrington, L.B., Bruyne, J.T.D., Kien, D.H.T., Hoang, N.L.T., *et al.* (2016) Establishment of a Wolbachia Superinfection in Aedes aegypti Mosquitoes as a Potential Approach for Future Resistance Management. *PLOS Pathogens*, **12**, e1005434.

Joyce, J.D., Nogueira, J.R., Bales, A.A., Pittman, K.E. & Anderson, J.R. (2011) Interactions between La Crosse virus and bacteria isolated from the digestive tract of Aedes albopictus (Diptera: Culicidae). *Journal of Medical Entomology*, **48**, 389–394.

Jupatanakul, N., Sim, S., Angleró-Rodríguez, Y.I., Souza-Neto, J., Das, S., Poti, K.E., *et al.* (2017) Engineered Aedes aegypti JAK/STAT Pathway-Mediated Immunity to Dengue Virus. *PLOS Neglected Tropical Diseases*, **11**, e0005187.

Jupatanakul, N., Sim, S. & Dimopoulos, G. (2014) The Insect Microbiome Modulates Vector Competence for Arboviruses. *Viruses*, **6**, 4294–4313.

Kambris, Z., Blagborough, A.M., Pinto, S.B., Blagrove, M.S.C., Godfray, H.C.J., Sinden, R.E., *et al.* (2010) Wolbachia Stimulates Immune Gene Expression and Inhibits Plasmodium Development in Anopheles gambiae. *PLOS Pathogens*, **6**, e1001143.

Kambris, Z., Cook, P.E., Phuc, H.K. & Sinkins, S.P. (2009) Immune Activation by Life-Shortening Wolbachia and Reduced Filarial Competence in Mosquitoes. *Science*, **326**, 134–136.

Keene, K.M., Foy, B.D., Sanchez-Vargas, I., Beaty, B.J., Blair, C.D. & Olson, K.E. (2004) RNA interference acts as a natural antiviral response to O'nyong-nyong virus (Alphavirus; Togaviridae) infection of Anopheles gambiae. *Proceedings of the National Academy of Sciences*, **101**, 17240–17245.

Kenney, J.L., Solberg, O.D., Langevin, S.A. & Brault, A.C. (2014) Characterization of a novel insect-specific flavivirus from Brazil: potential for inhibition of infection of arthropod cells with medically important flaviviruses. *The Journal of general virology*, **95**, 2796–2808.

Kent, M., Davis, J.R., Beier, J.C., Pumpuni, C.B. & Demaio, J. (1996) Bacterial Population Dynamics in Three Anopheline Species: The Impact on Plasmodium Sporogonic Development. *The American Journal of Tropical Medicine and Hygiene*, **54**, 214–218.

Kilpatrick, A.M., Ebel, G.D., Reddy, M.R., Fonseca, D.M. & Kramer, L.D. (2010) Spatial and Temporal Variation in Vector Competence of Culex pipiens and Cx. restuans Mosquitoes for West Nile Virus. *The American Journal of Tropical Medicine and Hygiene*, **83**, 607–613.

Kim, K.-H. (2006) Sphingobacterium daejeonense sp. nov., isolated from a compost sample. *INTERNATIONAL JOURNAL OF SYSTEMATIC AND EVOLUTIONARY MICROBIOLOGY*, **56**, 2031–2036.

Klindworth, A., Pruesse, E., Schweer, T., Peplies, J., Quast, C., Horn, M., *et al.* (2013) Evaluation of general 16S ribosomal RNA gene PCR primers for classical and next-generation sequencing-based diversity studies. *Nucleic Acids Research*, **41**, e1–e1.

Koenraadt, C.J., Kormaksson, M. & Harrington, L.C. (2010) Effects of inbreeding and genetic modification on Aedes aegypti larval competition and adult energy reserves. *Parasites & Vectors*, **3**, 92.

Kozomara, A. & Griffiths-Jones, S. (2014) miRBase: annotating high confidence microRNAs using deep sequencing data. *Nucleic Acids Research*, **42**, D68–D73.

Krüger, J. & Rehmsmeier, M. (2006) RNAhybrid: microRNA target prediction easy, fast and flexible. *Nucleic Acids Research*, **34**, W451–W454.

Kukutla, P., Lindberg, B.G., Pei, D., Rayl, M., Yu, W., Steritz, M., *et al.* (2014) Insights from the Genome Annotation of Elizabethkingia anophelis from the Malaria Vector Anopheles gambiae. *PLOS ONE*, **9**, e97715.

Kumar, S., Molina-Cruz, A., Gupta, L., Rodrigues, J. & Barillas-Mury, C. (2010) A peroxidase/dual oxidase system modulates midgut epithelial immunity in Anopheles gambiae. *Science (New York, N.Y.)*, **327**, 1644–1648.

Kuno, G. (2010) Early History of Laboratory Breeding of Aedes aegypti (Diptera: Culicidae) Focusing on the Origins and Use of Selected Strains. *Journal of Medical Entomology*, **47**, 957–971.

Kuwata, R., Isawa, H., Hoshino, K., Sasaki, T., Kobayashi, M., Maeda, K., *et al.* (2015) Analysis of Mosquito-Borne Flavivirus Superinfection in Culex tritaeniorhynchus (Diptera: Culicidae) Cells Persistently Infected with Culex Flavivirus (Flaviviridae). *Journal of Medical Entomology*, **52**, 222–229.

Lee, F. (2018) Differential carbohydrate utilization and organic acid production by honey bee symbionts, 29.

Lee, M., Etebari, K., Hall-Mendelin, S., Hurk, A.F. van den, Hobson-Peters, J., Vatipally, S., *et al.* (2017) Understanding the role of microRNAs in the interaction of Aedes aegypti mosquitoes with an insect-specific flavivirus. *Journal of General Virology*, **98**, 1892–1903.

Lemaitre, B., Nicolas, E., Michaut, L., Reichhart, J.-M. & Hoffmann, J.A. (1996) The Dorsoventral Regulatory Gene Cassette spätzle/Toll/cactus Controls the Potent Antifungal Response in Drosophila Adults. *Cell*, **86**, 973–983.

Leonard, J.N. & Schaffer, D.V. (2005) Computational Design of Antiviral RNA Interference Strategies That Resist Human Immunodeficiency Virus Escape. *Journal of Virology*, **79**, 1645–1654.

Lewis, Z. & Lizé, A. (2015) Insect behaviour and the microbiome. *Current Opinion in Insect Science*, Pests and resistance * Behavioural ecology, **9**, 86–90.

Li, X., Zhang, F., Coates, B., Zhang, Y., Zhou, X. & Cheng, D. (2016a) Comparative profiling of microRNAs in the winged and wingless English grain aphid, *Sitobion avenae* (F.) (Homoptera: Aphididae). *Scientific Reports*, **6**, 35668.

Li, Y., Song, L., Guo, M., Wang, L. & Liang, W. (2016b) Sphingobacterium populi sp. nov., isolated from bark of Populus × euramericana. *International Journal of Systematic and Evolutionary Microbiology*, **66**, 3456–3462.

Lima, A., Lovin, D.D., Hickner, P.V. & Severson, D.W. (2016) Evidence for an Overwintering Population of Aedes aegypti in Capitol Hill Neighborhood, Washington, DC. *The American Journal of Tropical Medicine and Hygiene*, **94**, 231–235.

Linenberg, I., Christophides, G.K. & Gendrin, M. (2016) Larval diet affects mosquito development and permissiveness to Plasmodium infection. *Scientific Reports*, **6**.

Liu, P., Li, X., Gu, J., Dong, Y., Liu, Y., Santhosh, P., *et al.* (2016a) Development of non-defective recombinant densovirus vectors for microRNA delivery in the invasive vector mosquito, *Aedes albopictus*. *Scientific Reports*, **6**, 20979.

Liu, Y., Zhou, Y., Wu, J., Zheng, P., Li, Y., Zheng, X., *et al.* (2015) The expression profile of Aedes albopictus miRNAs is altered by dengue virus serotype-2 infection. *Cell & Bioscience*, **5**, 16.

Liu, Y.-X., Li, F.-X., Liu, Z.-Z., Jia, Z.-R., Zhou, Y.-H., Zhang, H., *et al.* (2016b) Integrated analysis of miRNAs and transcriptomes in Aedes albopictus midgut reveals the differential expression profiles of immune-related genes during dengue virus serotype-2 infection. *Insect Science*, **23**, 377–385.

Livak, K.J. & Schmittgen, T.D. (2001) Analysis of Relative Gene Expression Data Using Real-Time Quantitative PCR and the 2−ΔΔCT Method. *Methods*, **25**, 402–408.

Lohaus, G. & Schwerdtfeger, M. (2014) Comparison of Sugars, Iridoid Glycosides and Amino Acids in Nectar and Phloem Sap of Maurandya barclayana, Lophospermum erubescens, and Brassica napus. *PLoS ONE*, **9**, e87689.

Lu, P., Bian, G., Pan, X. & Xi, Z. (2012) Wolbachia Induces Density-Dependent Inhibition to Dengue Virus in Mosquito Cells. *PLoS Neglected Tropical Diseases*, **6**, e1754.

Lucas, K.J., Myles, K.M. & Raikhel, A.S. (2013) Small RNAs: a new frontier in mosquito biology. *Trends in Parasitology*, **29**, 295–303.

Magnarelli, L.A. (1978) Nectar-Feeding by Female Mosquitoes and its Relation to Follicular Development and Parity. *Journal of Medical Entomology*, **14**, 527–530.

Maltz, M.A., Weiss, B.L., O'Neill, M., Wu, Y. & Aksoy, S. (2012) OmpA-Mediated Biofilm Formation Is Essential for the Commensal Bacterium Sodalis glossinidius To Colonize the Tsetse Fly Gut. *Appl. Environ. Microbiol.*, **78**, 7760–7768.

Mamlouk, D. & Gullo, M. (2013) Acetic Acid Bacteria: Physiology and Carbon Sources Oxidation. *Indian Journal of Microbiology*, **53**, 377–384.

Mancini, M.V., Spaccapelo, R., Damiani, C., Accoti, A., Tallarita, M., Petraglia, E., *et al.* (2016) Paratransgenesis to control malaria vectors: a semi-field pilot study. *Parasites & Vectors*, **9**, 140.

Manda, H., Gouagna, L.C., Foster, W.A., Jackson, R.R., Beier, J.C., Githure, J.I., *et al.* (2007) Effect of discriminative plant-sugar feeding on the survival and fecundity of Anopheles gambiae. *Malaria Journal*, **6**, 113.

Mandal, S., Van Treuren, W., White, R.A., Eggesbø, M., Knight, R. & Peddada, S.D. (2015) Analysis of composition of microbiomes: a novel method for studying microbial composition. *Microbial Ecology in Health & Disease*, **26**.

Mandell, D.J., Lajoie, M.J., Mee, M.T., Takeuchi, R., Kuznetsov, G., Norville, J.E., *et al.* (2015) Biocontainment of genetically modified organisms by synthetic protein design. *Nature*, **518**, 55–60.

Marti, G.A., Lastra, C.C.L., Pelizza, S.A. & García, J.J. (2006) Isolation of Paecilomyces lilacinus (Thom) Samson (Ascomycota: Hypocreales) from the Chagas disease vector, Triatoma infestans Klug (Hemiptera: Reduviidae) in an endemic area in Argentina. *Mycopathologia*, **162**, 369–372.

Martin, E., Bongiorno, G., Giovati, L., Montagna, M., Crotti, E., Damiani, C., *et al.* (2016) Isolation of a Wickerhamomyces anomalus yeast strain from the sandfly Phlebotomus perniciosus, displaying the killer phenotype. *Medical and Veterinary Entomology*, **30**, 101–106.

Martinez-Ibarra, J.A., Rodriguez, M.H., Arredondo-Jimenez, J.I. & Yuval, B. (1997) Influence of plant abundance on nectar feeding by Aedes aegypti (Diptera: Culicidae) in southern Mexico. *Journal of medical entomology*, **34**, 589–593.

McFarlane, M., Arias-Goeta, C., Martin, E., O'Hara, Z., Lulla, A., Mousson, L., *et al.* (2014) Characterization of Aedes aegypti Innate-Immune Pathways that Limit Chikungunya Virus Replication. *PLoS Neglected Tropical Diseases*, **8**, e2994.

McNaughton, D. (2012) The Importance of Long-Term Social Research in Enabling Participation and Developing Engagement Strategies for New Dengue Control Technologies. *PLOS Neglected Tropical Diseases*, **6**, e1785.

McNaughton, D. & Duong, T.T.H. (2014) Designing a Community Engagement Framework for a New Dengue Control Method: A Case Study from Central Vietnam. *PLOS Neglected Tropical Diseases*, **8**, e2794.

Medlock, J., Atkins, K.E., Thomas, D.N., Aksoy, S. & Galvani, A.P. (2013) Evaluating Paratransgenesis as a Potential Control Strategy for African Trypanosomiasis. *PLOS Neglected Tropical Diseases*, **7**, e2374.

Menchaca, A.C., Visi, D.K., Strey, O.F., Teel, P.D., Kalinowski, K., Allen, M.S., *et al.* (2013) Preliminary Assessment of Microbiome Changes Following Blood-Feeding and Survivorship in the Amblyomma americanum Nymph-to-Adult Transition using Semiconductor Sequencing. *PLOS ONE*, **8**, e67129.

Miesen, P., Girardi, E. & Rij, R.P. van. (2015) Distinct sets of PIWI proteins produce arbovirus and transposon-derived piRNAs in Aedes aegypti mosquito cells. *Nucleic Acids Research*, **43**, 6545–6556.

Miesen, P., Ivens, A., Buck, A.H. & Rij, R.P. van. (2016) Small RNA Profiling in Dengue Virus 2-Infected Aedes Mosquito Cells Reveals Viral piRNAs and Novel Host miRNAs. *PLOS Neglected Tropical Diseases*, **10**, e0004452.

Minard, G., Tran, F.H., Raharimalala, F.N., Hellard, E., Ravelonandro, P., Mavingui, P., *et al.* (2013) Prevalence, genomic and metabolic profiles of Acinetobacter and Asaia associated with field-caught Aedes albopictus from Madagascar. *FEMS Microbiology Ecology*, **83**, 63–73.

Minard, G., Tran, F.-H., Tran Van, V., Fournier, C., Potier, P., Roiz, D., *et al.* (2018) Shared larval rearing environment, sex, female size and genetic diversity shape Ae. albopictus bacterial microbiota. *PLOS ONE*, **13**, e0194521.

Miranda, K.C., Huynh, T., Tay, Y., Ang, Y.-S., Tam, W.-L., Thomson, A.M., *et al.* (2006) A Pattern-Based Method for the Identification of MicroRNA Binding Sites and Their Corresponding Heteroduplexes. *Cell*, **126**, 1203–1217.

Moghadam, N.N., Thorshauge, P.M., Kristensen, T.N., Jonge, N. de, Bahrndorff, S., Kjeldal, H., *et al.* (2017) Strong responses of Drosophila melanogaster microbiota to developmental temperature. *Fly*, **12**, 1–12.

Mohanty, A.K., Balabaskaran Nina, P., Ballav, S., Vernekar, S., Parkar, S., D'souza, M., *et al.* (2018) Susceptibility of wild and colonized Anopheles stephensi to Plasmodium vivax infection. *Malaria Journal*, **17**.

Moll, R.M., Romoser, W.S., Modrzakowski, M.C., Moncayo, A.C. & Lerdthusnee, K. (2001) Meconial peritrophic membranes and the fate of midgut bacteria during mosquito (Diptera: Culicidae) metamorphosis. *Journal of Medical Entomology*, **38**, 29–32.

Molloy, J.C., Sommer, U., Viant, M.R. & Sinkins, S.P. (2016) Wolbachia Modulates Lipid Metabolism in Aedes albopictus Mosquito Cells. *Applied and Environmental Microbiology*, **82**, 3109–3120.

Moran, N.A. (2015) Genomics of the honey bee microbiome. *Current Opinion in Insect Science*, **10**, 22–28.

Morazzani, E.M., Wiley, M.R., Murreddu, M.G., Adelman, Z.N. & Myles, K.M. (2012) Production of Virus-Derived Ping-Pong-Dependent piRNA-like Small RNAs in the Mosquito Soma. *PLOS Pathogens*, **8**, e1002470.

Moreira, L.A., Iturbe-Ormaetxe, I., Jeffery, J.A., Lu, G., Pyke, A.T., Hedges, L.M., *et al.* (2009) A Wolbachia Symbiont in Aedes aegypti Limits Infection with Dengue, Chikungunya, and Plasmodium. *Cell*, **139**, 1268–1278.

Mostowy, W.M. & Foster, W.A. (2004) Antagonistic effects of energy status on meal size and egg-batch size of Aedes aegypti (Diptera: Culicidae). *Journal of Vector Ecology: Journal of the Society for Vector Ecology*, **29**, 84–93.

Mota, F.F. da, Marinho, L.P., Moreira, C.J. de C., Lima, M.M., Mello, C.B., Garcia, E.S., *et al.* (2012) Cultivation-Independent Methods Reveal Differences among Bacterial Gut Microbiota in Triatomine Vectors of Chagas Disease. *PLoS Neglected Tropical Diseases*, **6**, e1631.

Mourya, D.T., Pidiyar, V., Patole, M., Gokhale, M.D. & Shouche, Y. (2002) Effect of Midgut Bacterial Flora of Aedes aegypti on the Susceptibility of Mosquitoes to Dengue Viruses, **26**, 5.

Mourya, D.T. & Soman, R.S. (1985) Effect of gregarine parasite, Ascogregarina culicis & tetracycline on the susceptibility of Culex bitaeniorhynchus to JE virus. *The Indian journal of medical research*, **81**, 247–250.

Mousson, L., Martin, E., Zouache, K., Madec, Y., Mavingui, P. & Failloux, A.B. (2010) Wolbachia modulates Chikungunya replication in Aedes albopictus. *Molecular Ecology*, **19**, 1953–1964.

Mousson, L., Zouache, K., Arias-Goeta, C., Raquin, V., Mavingui, P. & Failloux, A.-B. (2012) The Native Wolbachia Symbionts Limit Transmission of Dengue Virus in Aedes albopictus. *PLOS Neglected Tropical Diseases*, **6**, e1989.

Muhammad, A., Fang, Y., Hou, Y. & Shi, Z. (2017) The Gut Entomotype of Red Palm Weevil Rhynchophorus ferrugineus Olivier (Coleoptera: Dryophthoridae) and Their Effect on Host Nutrition Metabolism. *Frontiers in Microbiology*, **8**.

Murdock, C.C., Blanford, S., Hughes, G.L., Rasgon, J.L. & Thomas, M.B. (2014) Temperature alters *Plasmodium* blocking by *Wolbachia*. *Scientific Reports*, **4**, 3932.

Murdock, C.C., Paaijmans, K.P., Cox-Foster, D., Read, A.F. & Thomas, M.B. (2012) Rethinking vector immunology: the role of environmental temperature in shaping resistance. *Nature Reviews Microbiology*, **10**, 869–876.

Murphy, K.A., Tabuloc, C.A., Cervantes, K.R. & Chiu, J.C. (2016) Ingestion of genetically modified yeast symbiont reduces fitness of an insect pest via RNA interference. *Scientific Reports*, **6**, 22587.

Murray, J.V., Jansen, C.C. & De Barro, P. (2016) Risk Associated with the Release of Wolbachia-Infected Aedes aegypti Mosquitoes into the Environment in an Effort to Control Dengue. *Frontiers in Public Health*, **4**.

Muturi, E.J., Bara, J.J., Rooney, A.P. & Hansen, A.K. (2016) Midgut fungal and bacterial microbiota of *Aedes triseriatus* and *Aedes japonicus* shift in response to La Crosse virus infection. *Molecular Ecology*.

Muturi, E.J., Lagos-Kutz, D., Dunlap, C., Ramirez, J.L., Rooney, A.P., Hartman, G.L., *et al.* (2018) Mosquito microbiota cluster by host sampling location. *Parasites & Vectors*, **11**.

Mwadondo, style="text-align: justify;"> E.M., Amanuel, G., Amos, E.A. & Remmy, W.K. (2017) Midgut bacterial diversity analysis of laboratory reared and wild Anopheles gambiae and Culex quinquefasciatus mosquitoes in Kenya. *African Journal of Microbiology Research*, **11**, 1171–1183.

Naqqash, M.N., Gökçe, A., Bakhsh, A. & Salim, M. (2016) Insecticide resistance and its molecular basis in urban insect pests. *Parasitology Research*, **115**, 1363–1373.

Narasimhan, S., Rajeevan, N., Liu, L., Zhao, Y.O., Heisig, J., Pan, J., *et al.* (2014) Gut Microbiota of the Tick Vector Ixodes scapularis Modulate Colonization of the Lyme Disease Spirochete. *Cell Host & Microbe*, **15**, 58–71.

Nasar, F., Gorchakov, R.V., Tesh, R.B. & Weaver, S.C. (2015) Eilat Virus Host Range Restriction Is Present at Multiple Levels of the Virus Life Cycle. *Journal of Virology*, **89**, 1404–1418.

Nelson, M.J. (1986) Aedes aegypti: biology and ecology.

Newman, C.M., Cerutti, F., Anderson, T.K., Hamer, G.L., Walker, E.D., Kitron, U.D., *et al.* (2011) Culex Flavivirus and West Nile Virus Mosquito Coinfection and Positive Ecological Association in Chicago, United States. *Vector-Borne and Zoonotic Diseases*, **11**, 1099–1105.

Ng, T.F.F., Willner, D.L., Lim, Y.W., Schmieder, R., Chau, B., Nilsson, C., *et al.* (2011) Broad Surveys of DNA Viral Diversity Obtained through Viral Metagenomics of Mosquitoes. *PLOS ONE*, **6**, e20579.

Noden, B.H., Vaughan, J.A., Pumpuni, C.B. & Beier, J.C. (2011) Mosquito ingestion of antibodies against mosquito midgut microbiota improves conversion of ookinetes to oocysts for Plasmodium falciparum, but not P. yoelii. *Parasitology International*, **60**, 440–446.

O'Connor, L., Plichart, C., Sang, A.C., Brelsfoard, C.L., Bossin, H.C. & Dobson, S.L. (2012) Open Release of Male Mosquitoes Infected with a Wolbachia Biopesticide: Field Performance and Infection Containment. *PLOS Neglected Tropical Diseases*, **6**, e1797.

Ohta, A. & Nishiyama, Y. (2011) Mitochondria and viruses. *Mitochondrion*, **11**, 1–12.

Oliveira, J.H.M., Gonçalves, R.L.S., Lara, F.A., Dias, F.A., Gandara, A.C.P., Menna-Barreto, R.F.S., *et al.* (2011) Blood Meal-Derived Heme Decreases ROS Levels in the Midgut of Aedes aegypti and Allows Proliferation of Intestinal Microbiota. *PLOS Pathogens*, **7**, e1001320.

Olson, K.E. & Blair, C.D. (2015) Arbovirus–mosquito interactions: RNAi pathway. *Current Opinion in Virology*, SI: 15 Virus–vector interactions•Viral immunology, **15**, 119–126.

Osei-Poku, J., Mbogo, C.M., Palmer, W.J. & Jiggins, F.M. (2012) Deep sequencing reveals extensive variation in the gut microbiota of wild mosquitoes from Kenya. *Molecular Ecology*, **21**, 5138–5150.

Pan, X., Zhou, G., Wu, J., Bian, G., Lu, P., Raikhel, A.S., *et al.* (2012) Wolbachia induces reactive oxygen species (ROS)-dependent activation of the Toll pathway to control dengue virus in the mosquito Aedes aegypti. *Proceedings of the National Academy of Sciences*, **109**, E23–E31.

Pang, X., Xiao, X., Liu, Y., Zhang, R., Liu, J., Liu, Q., *et al.* (2016) Mosquito C-type lectins maintain gut microbiome homeostasis. *Nature microbiology*, **1**.

Pei, D., Jiang, J., Yu, W., Kukutla, P., Uentillie, A. & Xu, J. (2015) The waaL gene mutation compromised the inhabitation of Enterobacter sp. Ag1 in the mosquito gut environment. *Parasites & Vectors*, **8**.

Pennington, M.J., Prager, S.M., Walton, W.E. & Trumble, J.T. (2016) Culex quinquefasciatus larval microbiomes vary with instar and exposure to common wastewater contaminants. *Scientific Reports*, **6**.

Perera, R., Riley, C., Isaac, G., Hopf-Jannasch, A.S., Moore, R.J., Weitz, K.W., *et al.* (2012) Dengue Virus Infection Perturbs Lipid Homeostasis in Infected Mosquito Cells. *PLoS Pathogens*, **8**, e1002584.

Pijlman, G.P. (2014) Flavivirus RNAi suppression: decoding non-coding RNA. *Current Opinion in Virology*, Virus-glycan interactions and pathogenesis / Viruses and RNA interference, **7**, 55–60.

Pollock, J., Glendinning, L., Wisedchanwet, T. & Watson, M. (2018) The Madness of Microbiome: Attempting To Find Consensus "Best Practice" for 16S Microbiome Studies. *Appl. Environ. Microbiol.*, **84**, e02627-17.

Pompon, J., Morales-Vargas, R., Manuel, M., Huat Tan, C., Vial, T., Hao Tan, J., *et al.* (2017) A Zika virus from America is more efficiently transmitted than an Asian virus by Aedes aegypti mosquitoes from Asia. *Scientific Reports*, **7**.

Qualls, W.A., Naranjo, D.P., Subía, M.A., Ramon, G., Cevallos, V., Grijalva, I., *et al.* (2016) Movement of Aedes aegypti following a sugar meal and its implication in the development of control strategies in Durán, Ecuador. *Journal of Vector Ecology*, **41**, 224–231.

Quast, C., Pruesse, E., Yilmaz, P., Gerken, J., Schweer, T., Yarza, P., *et al.* (2012) The SILVA ribosomal RNA gene database project: improved data processing and web-based tools. *Nucleic Acids Research*, **41**, D590–D596.

Ramirez, J.L. & Dimopoulos, G. (2010) The Toll immune signaling pathway control conserved anti-dengue defenses across diverse Ae. aegypti strains and against multiple dengue virus serotypes. *Developmental & Comparative Immunology*, **34**, 625–629.

Ramirez, J.L., Short, S.M., Bahia, A.C., Saraiva, R.G., Dong, Y., Kang, S., *et al.* (2014) Chromobacterium Csp_P Reduces Malaria and Dengue Infection in Vector Mosquitoes and Has Entomopathogenic and In Vitro Anti-pathogen Activities. *PLoS Pathogens*, **10**, e1004398.

Ramirez, J.L., Souza-Neto, J., Torres Cosme, R., Rovira, J., Ortiz, A., Pascale, J.M., *et al.* (2012) Reciprocal tripartite interactions between the Aedes aegypti midgut microbiota, innate immune system and dengue virus influences vector competence. *PLoS Neglected Tropical Diseases*, **6**, e1561.

Rani, A., Sharma, A., Rajagopal, R., Adak, T. & Bhatnagar, R.K. (2009) Bacterial diversity analysis of larvae and adult midgut micro-flora using culture-dependent and culture-independent methods in lab-reared and field-collected Anopheles stephensi-an Asian malarial vector. *BMC Microbiology*, **9**, 96.

Ranson, H. & Lissenden, N. (2016) Insecticide Resistance in African Anopheles Mosquitoes: A Worsening Situation that Needs Urgent Action to Maintain Malaria Control. *Trends in Parasitology*, Special Issue: Vectors, **32**, 187–196.

Rasmussen, S.A., Jamieson, D.J., Honein, M.A. & Petersen, L.R. (2016) Zika Virus and Birth Defects — Reviewing the Evidence for Causality [WWW Document]. *https://doi.org/10.1056/NEJMsr1604338*. URL https://www.nejm.org/doi/10.1056/NEJMsr1604338?url_ver=Z39.88-2003&rfr_id=ori%3Arid%3Acrossref.org&rfr_dat=cr_pub%3Dwww.ncbi.nlm.nih.gov [accessed on 13 April 2016].

Ren, X., Hoiczyk, E. & Rasgon, J.L. (2008) Viral Paratransgenesis in the Malaria Vector Anopheles gambiae. *PLOS Pathogens*, **4**, e1000135.

Ren, X., Hughes, G.L., Niu, G., Suzuki, Y. & Rasgon, J.L. (2014) Anopheles gambiae densovirus (AgDNV) has negligible effects on adult survival and transcriptome of its mosquito host. *PeerJ*, **2**, e584.

Ricci, I., Damiani, C., Scuppa, P., Mosca, M., Crotti, E., Rossi, P., *et al.* (2011a) The yeast Wickerhamomyces anomalus (Pichia anomala) inhabits the midgut and reproductive system of the Asian malaria vector Anopheles stephensi. *Environmental Microbiology*, **13**, 911–921.

Ricci, I., Mosca, M., Valzano, M., Damiani, C., Scuppa, P., Rossi, P., *et al.* (2011b) Different mosquito species host Wickerhamomyces anomalus (Pichia anomala): perspectives on vector-borne diseases symbiotic control. *Antonie van Leeuwenhoek*, **99**, 43–50.

Riehle, M.A., Moreira, C.K., Lampe, D., Lauzon, C. & Jacobs-Lorena, M. (2007) Using bacteria to express and display anti-Plasmodium molecules in the mosquito midgut. *International journal for parasitology*, **37**, 595–603.

Roby, J.A., Pijlman, G.P., Wilusz, J. & Khromykh, A.A. (2014) Noncoding Subgenomic Flavivirus RNA: Multiple Functions in West Nile Virus Pathogenesis and Modulation of Host Responses. *Viruses*, **6**, 404–427.

Rodrigues, J., Brayner, F.A., Alves, L.C., Dixit, R. & Barillas-Mury, C. (2010) Hemocyte Differentiation Mediates Innate Immune Memory in Anopheles gambiae Mosquitoes. *Science*, **329**, 1353–1355.

Rodríguez-Riaño, T., Ortega-Olivencia, A., López, J., Pérez-Bote, J.L. & Navarro-Pérez, M.L. (2014) Main sugar composition of floral nectar in three species groups of *Scrophularia* (Scrophulariaceae) with different principal pollinators. *Plant Biology*, n/a-n/a.

Romoli, O. & Gendrin, M. (2018) The tripartite interactions between the mosquito, its microbiota and Plasmodium. *Parasites & Vectors*, **11**.

Ross, P.A., Endersby-Harshman, N.M. & Hoffmann, A.A. (2018) A comprehensive assessment of inbreeding and laboratory adaptation in *Aedes aegypti* mosquitoes. *Evolutionary Applications*.

Rossi, P., Ricci, I., Cappelli, A., Damiani, C., Ulissi, U., Mancini, M.V., *et al.* (2015) Mutual exclusion of Asaia and Wolbachia in the reproductive organs of mosquito vectors. *Parasites & Vectors*, **8**, 278.

Roundy, C.M., Azar, S.R., Rossi, S.L., Huang, J.H., Leal, G., Yun, R., *et al.* (2017a) Variation in *Aedes aegypti* Mosquito Competence for Zika Virus Transmission. *Emerging Infectious Diseases*, **23**, 625–632.

Roundy, C.M., Azar, S.R., Rossi, S.L., Huang, J.H., Leal, G., Yun, R., *et al.* (2017b) Variation in *Aedes aegypti* Mosquito Competence for Zika Virus Transmission. *Emerging Infectious Diseases*, **23**, 625–632.

Rovner, A.J., Haimovich, A.D., Katz, S.R., Li, Z., Grome, M.W., Gassaway, B.M., *et al.* (2015) Recoded organisms engineered to depend on synthetic amino acids. *Nature*, **518**, 89–93.

Rueda, A., Barturen, G., Lebrón, R., Gómez-Martín, C., Alganza, Á., Oliver, J.L., *et al.* (2015) sRNAtoolbox: an integrated collection of small RNA research tools. *Nucleic Acids Research*, **43**, W467–W473.

Sakamoto, J.M., Ng, T.F.F., Suzuki, Y., Tsujimoto, H., Deng, X., Delwart, E., *et al.* (2016) Bunyaviruses are common in male and female Ixodes scapularis ticks in central Pennsylvania. *PeerJ*, **4**, e2324.

Saldaña, M.A., Etebari, K., Hart, C.E., Widen, S.G., Wood, T.G., Thangamani, S., *et al.* (2017) Zika virus alters the microRNA expression profile and elicits an RNAi response in Aedes aegypti mosquitoes. *PLOS Neglected Tropical Diseases*, **11**, e0005760.

Sánchez-Vargas, I., Scott, J.C., Poole-Smith, B.K., Franz, A.W.E., Barbosa-Solomieu, V., Wilusz, J., *et al.* (2009) Dengue Virus Type 2 Infections of Aedes aegypti Are Modulated by the Mosquito's RNA Interference Pathway. *PLoS Pathogens*, **5**, e1000299.

Schlein, Y. & Müller, G.C. (2015) Decrease of larval and subsequent adult Anopheles sergentii populations following feeding of adult mosquitoes from Bacillus sphaericus-containing attractive sugar baits. *Parasites & Vectors*, **8**.

Schlein, Y., Polacheck, I. & Yuval, B. (1985) Mycoses, bacterial infections and antibacterial activity in sandifies (Psychodidae) and their possible role in the transmission of leishmaniasis. *Parasitology*, **90**, 57–66.

Schnettler, E., Donald, C.L., Human, S., Watson, M., Siu, R.W.C., McFarlane, M., *et al.* (2013a) Knockdown of piRNA pathway proteins results in enhanced Semliki Forest virus production in mosquito cells. *Journal of General Virology*, **94**, 1680–1689.

Schnettler, E., Ratinier, M., Watson, M., Shaw, A.E., McFarlane, M., Varela, M., *et al.* (2013b) RNA Interference Targets Arbovirus Replication in Culicoides Cells. *Journal of Virology*, **87**, 2441–2454.

Schnettler, E., Sreenu, V.B., Mottram, T. & McFarlane, M. (2016) Wolbachia restricts insect-specific flavivirus infection in Aedes aegypti cells. *The Journal of General Virology*, **97**, 3024–3029.

Schnettler, E., Sterken, M.G., Leung, J.Y., Metz, S.W., Geertsema, C., Goldbach, R.W., *et al.* (2012) Noncoding Flavivirus RNA Displays RNA Interference Suppressor Activity in Insect and Mammalian Cells. *Journal of Virology*, **86**, 13486–13500.

Schnitger, A.K.D., Kafatos, F.C. & Osta, M.A. (2007) The Melanization Reaction Is Not Required for Survival of *Anopheles gambiae* Mosquitoes after Bacterial Infections. *Journal of Biological Chemistry*, **282**, 21884–21888.

Scott, J.C., Brackney, D.E., Campbell, C.L., Bondu-Hawkins, V., Hjelle, B., Ebel, G.D., *et al.* (2010) Comparison of Dengue Virus Type 2-Specific Small RNAs from RNA Interference-Competent and –Incompetent Mosquito Cells. *PLoS Neglected Tropical Diseases*, **4**, e848.

Segata, N., Baldini, F., Pompon, J., Garrett, W.S., Truong, D.T., Dabiré, R.K., *et al.* (2016) The reproductive tracts of two malaria vectors are populated by a core microbiome and by gender- and swarm-enriched microbial biomarkers. *Scientific Reports*, **6**, 24207.

Shan, C., Xie, X., Muruato, A.E., Rossi, S.L., Roundy, C.M., Azar, S.R., *et al.* (2016) An Infectious cDNA Clone of Zika Virus to Study Viral Virulence, Mosquito Transmission, and Antiviral Inhibitors. *Cell Host & Microbe*, **19**, 891–900.

Shannon, C.E. (1948) A Mathematical Theory of Communication. *Bell System Technical Journal*, **27**, 379–423.

Sharma, P., Sharma, S., Maurya, R.K., De, T.D., Thomas, T., Lata, S., *et al.* (2014) Salivary glands harbor more diverse microbial communities than gut in Anopheles culicifacies. *Parasites & Vectors*, **7**, 235.

Sharon, G., Segal, D., Ringo, J.M., Hefetz, A., Zilber-Rosenberg, I. & Rosenberg, E. (2010) Commensal bacteria play a role in mating preference of Drosophila melanogaster. *Proceedings of the National Academy of Sciences*, **107**, 20051–20056.

Shaw, W.R., Marcenac, P., Childs, L.M., Buckee, C.O., Baldini, F., Sawadogo, S.P., *et al.* (2016) *Wolbachia* infections in natural *Anopheles* populations affect egg laying and negatively correlate with *Plasmodium* development. *Nature Communications*, **7**, 11772.

Shrinet, J., Jain, S., Jain, J., Bhatnagar, R.K. & Sunil, S. (2014) Next Generation Sequencing Reveals Regulation of Distinct Aedes microRNAs during Chikungunya Virus Development. *PLOS Neglected Tropical Diseases*, **8**, e2616.

Shropshire, J.D. & Bordenstein, S.R. (2016) Speciation by Symbiosis: the Microbiome and Behavior. *mBio*, **7**, e01785-15.

Skalsky, R.L., Vanlandingham, D.L., Scholle, F., Higgs, S. & Cullen, B.R. (2010) Identification of microRNAs expressed in two mosquito vectors, Aedes albopictus and Culex quinquefasciatus. *BMC Genomics*, **11**, 119.

Snow, R.W., Guerra, C.A., Noor, A.M., Myint, H.Y. & Hay, S.I. (2005) The global distribution of clinical episodes of Plasmodium falciparum malaria. *Nature*, **434**, 214.

Soares, T.S., Buarque, D.S., Queiroz, B.R., Gomes, C.M., Braz, G.R.C., Araújo, R.N., *et al.* (2015) A Kazal-type inhibitor is modulated by Trypanosoma cruzi to control microbiota inside the anterior midgut of Rhodnius prolixus. *Biochimie*, **112**, 41–48.

Solé, M., Rius, N. & Lorén, J.G. (2000) Rapid extracellular acidification induced by glucose metabolism in non-proliferating cells of Serratia marcescens. *International Microbiology: The Official Journal of the Spanish Society for Microbiology*, **3**, 39–43.

Souza-Neto, J.A., Sim, S. & Dimopoulos, G. (2009) An evolutionary conserved function of the JAK-STAT pathway in anti-dengue defense. *Proceedings of the National Academy of Sciences*, **106**, 17841–17846.

Stathopoulos, S., Neafsey, D.E., Lawniczak, M.K.N., Muskavitch, M.A.T. & Christophides, G.K. (2014) Genetic Dissection of Anopheles gambiae Gut Epithelial Responses to Serratia marcescens. *PLOS Pathogens*, **10**, e1003897.

Stefano, G.B., Samuel, J. & Kream, R.M. (2017) Antibiotics May Trigger Mitochondrial Dysfunction Inducing Psychiatric Disorders. *Medical Science Monitor : International Medical Journal of Experimental and Clinical Research*, **23**, 101–106.

Steyn, A., Roets, F. & Botha, A. (2016) Yeasts Associated with Culex pipiens and Culex theileri Mosquito Larvae and the Effect of Selected Yeast Strains on the Ontogeny of Culex pipiens. *Microbial Ecology*, **71**, 747–760.

Straif, S.C., Mbogo, C.N.M., Toure, A.M., Walker, E.D., Kaufman, M., Toure, Y.T., *et al.* (1998) Midgut Bacteria in Anopheles gambiae and An. funestus (Diptera: Culicidae) from Kenya and Mali. *Journal of Medical Entomology*, **35**, 222–226.

Suzuki, Y., Niu, G., Hughes, G.L. & Rasgon, J.L. (2014) A viral over-expression system for the major malaria mosquito *Anopheles gambiae*. *Scientific Reports*, **4**, 5127.

Tabachnick, W.J., Wallis, G.P., Aitken, T.H., Miller, B.R., Amato, G.D., Lorenz, L., *et al.* (1985) Oral infection of Aedes aegypti with yellow fever virus: geographic variation and genetic considerations. *The American Journal of Tropical Medicine and Hygiene*, **34**, 1219–1224.

Taracena, M.L., Oliveira, P.L., Almendares, O., Umaña, C., Lowenberger, C., Dotson, E.M., *et al.* (2015) Genetically Modifying the Insect Gut Microbiota to Control Chagas Disease Vectors through Systemic RNAi. *PLOS Neglected Tropical Diseases*, **9**, e0003358.

Tchioffo, M.T., Boissière, A., Abate, L., Nsango, S.E., Bayibéki, A.N., Awono-Ambéné, P.H., *et al.* (2016) Dynamics of Bacterial Community Composition in the Malaria Mosquito's Epithelia. *Frontiers in Microbiology*, **6**.

Teixeira, L., Ferreira, Á. & Ashburner, M. (2008) The Bacterial Symbiont Wolbachia Induces Resistance to RNA Viral Infections in Drosophila melanogaster. *PLOS Biology*, **6**, e1000002.

Ten, L.N., Liu, Q.-M., Wan-Taek, I., Aslam, Z. & Lee, S.-T. (2006) Sphingobacterium composti sp. nov., a Novel DNase-Producing Bacterium Isolated from Compost. *Journal of Microbiology and Biotechnology*, **16**, 1728–1733.

Teo, J., Tan, S.Y.-Y., Liu, Y., Tay, M., Ding, Y., Li, Y., *et al.* (2014) Comparative Genomic Analysis of Malaria Mosquito Vector-Associated Novel Pathogen Elizabethkingia anophelis. *Genome Biology and Evolution*, **6**, 1158–1165.

Terenius, O., Lindh, J.M., Eriksson-Gonzales, K., Bussière, L., Laugen, A.T., Bergquist, H., *et al.* (2012) Midgut bacterial dynamics in Aedes aegypti. *FEMS Microbiology Ecology*, **80**, 556–565.

Terenius, O., Oliveira, C.D. de, Pinheiro, W.D., Tadei, W.P., James, A.A. & Marinotti, O. (2008) 16S rRNA Gene Sequences from Bacteria Associated with Adult Anopheles darlingi (Diptera: Culicidae) Mosquitoes. *Journal of Medical Entomology*, **45**, 172–175.

Tesh, R.B., Gubler, D.J. & Rosen, L. (1976) Variation among Geographic Strains of Aedes Albopictus in Susceptibility to Infection with Chikungunya Virus *. *The American Journal of Tropical Medicine and Hygiene*, **25**, 326–335.

Thailayil, J., Magnusson, K., Godfray, H.C.J., Crisanti, A. & Catteruccia, F. (2011) Spermless males elicit large-scale female responses to mating in the malaria mosquito Anopheles gambiae. *Proceedings of the National Academy of Sciences*, **108**, 13677–13681.

Timmons, L., Court, D.L. & Fire, A. (2001) Ingestion of bacterially expressed dsRNAs can produce specific and potent genetic interference in Caenorhabditis elegans.

Tokarz, R., Williams, S.H., Sameroff, S., Leon, M.S., Jain, K. & Lipkin, W.I. (2014) Virome Analysis of Amblyomma americanum, Dermacentor variabilis, and Ixodes scapularis Ticks Reveals Novel Highly Divergent Vertebrate and Invertebrate Viruses. *Journal of Virology*, **88**, 11480–11492.

Tol, S. van & Dimopoulos, G. (2016) Chapter Nine - Influences of the Mosquito Microbiota on Vector Competence. In *Advances in Insect Physiology*, Progress in Mosquito Research (ed. by Raikhel, A.S.). Academic Press, pp. 243–291.

Townsend, G.E., Han, W., Schwalm, N.D., Raghavan, V., Barry, N.A., Goodman, A.L., *et al.* (2019) Dietary sugar silences a colonization factor in a mammalian gut symbiont. *Proceedings of the National Academy of Sciences*, **116**, 233–238.

Trobaugh, D.W., Gardner, C.L., Sun, C., Haddow, A.D., Wang, E., Chapnik, E., *et al.* (2014) RNA viruses can hijack vertebrate microRNAs to suppress innate immunity. *Nature*, **506**, 245–248.

Valzano, M., Cecarini, V., Cappelli, A., Capone, A., Bozic, J., Cuccioloni, M., *et al.* (2016) A yeast strain associated to Anopheles mosquitoes produces a toxin able to kill malaria parasites. *Malaria Journal*, **15**.

VanHook, A.M. (2018) Antibiotics directly affect host cell metabolism. *Sci. Signal.*, **11**, eaas9172.

Vasilakis, N. & Tesh, R.B. (2015) Insect-specific viruses and their potential impact on arbovirus transmission. *Current Opinion in Virology*, SI: 15 Virus–vector interactions•Viral immunology, **15**, 69–74.

Velazquez, E.M., Nguyen, H., Heasley, K.T., Saechao, C.H., Gil, L.M., Rogers, A.W.L., *et al.* (2019) Endogenous Enterobacteriaceae underlie variation in susceptibility to Salmonella infection. *Nature Microbiology*, 1.

Veronesi, E., Paslaru, A., Silaghi, C., Tobler, K., Glavinic, U., Torgerson, P., *et al.* (2018) Experimental evaluation of infection, dissemination, and transmission rates for two West Nile virus strains in European Aedes japonicus under a fluctuating temperature regime. *Parasitology Research*, **117**, 1925–1932.

Vieira, C.S., Mattos, D.P., Waniek, P.J., Santangelo, J.M., Figueiredo, M.B., Gumiel, M., *et al.* (2015) Rhodnius prolixus interaction with Trypanosoma rangeli: modulation of the immune system and microbiota population. *Parasites & Vectors*, **8**, 135.

Vilcarromero, S., Chen, R., Suarez-Ognio, L., Johnson, W.L., Morrison, A.C., Long, K.C., *et al.* (2014) Lineage II of Southeast Asian/American DENV-2 is Associated with a Severe Dengue Outbreak in the Peruvian Amazon. *The American Journal of Tropical Medicine and Hygiene*, **91**, 611–620.

Villegas, L.E.M., Campolina, T.B., Barnabe, N.R., Orfano, A.S., Chaves, B.A., Norris, D.E., *et al.* (2018) Zika virus infection modulates the bacterial diversity associated with Aedes aegypti as revealed by metagenomic analysis. *PLOS ONE*, **13**, e0190352.

Vodovar, N., Bronkhorst, A.W., Cleef, K.W.R. van, Miesen, P., Blanc, H., Rij, R.P. van, *et al.* (2012) Arbovirus-Derived piRNAs Exhibit a Ping-Pong Signature in Mosquito Cells. *PLOS ONE*, **7**, e30861.

Walker, T., Johnson, P.H., Moreira, L.A., Iturbe-Ormaetxe, I., Frentiu, F.D., McMeniman, C.J., *et al.* (2011) The *w*Mel *Wolbachia* strain blocks dengue and invades caged *Aedes aegypti* populations. *Nature*, **476**, 450–453.

Wang, J., Brelsfoard, C., Wu, Y. & Aksoy, S. (2013a) Intercommunity effects on microbiome and GpSGHV density regulation in tsetse flies. *Journal of Invertebrate Pathology*, Tse Tse Fly Symposium, **112**, S32–S39.

Wang, J., Weiss, B.L. & Aksoy, S. (2013b) Tsetse fly microbiota: form and function. *Frontiers in Cellular and Infection Microbiology*, **3**.

Wang, J., Wu, Y., Yang, G. & Aksoy, S. (2009) Interactions between mutualist Wigglesworthia and tsetse peptidoglycan recognition protein (PGRP-LB) influence trypanosome transmission. *Proceedings of the National Academy of Sciences*, **106**, 12133–12138.

Wang, S., Dos-Santos, A.L.A., Huang, W., Liu, K.C., Oshaghi, M.A., Wei, G., *et al.* (2017) Driving mosquito refractoriness to Plasmodium falciparum with engineered symbiotic bacteria. *Science*, **357**, 1399–1402.

Wang, S., Ghosh, A.K., Bongio, N., Stebbings, K.A., Lampe, D.J. & Jacobs-Lorena, M. (2012) Fighting malaria with engineered symbiotic bacteria from vector mosquitoes. *Proceedings of the National Academy of Sciences*, **109**, 12734–12739.

Wang, S. & Jacobs-Lorena, M. (2013) Genetic approaches to interfere with malaria transmission by vector mosquitoes. *Trends in Biotechnology*, Special Issue: Celebrating 30 years of biotechnology, **31**, 185–193.

Wang, Y., Gilbreath, T.M., Kukutla, P., Yan, G. & Xu, J. (2011) Dynamic Gut Microbiome across Life History of the Malaria Mosquito Anopheles gambiae in Kenya. *PLoS ONE*, **6**, e24767.

Wang, Y.-H., Hu, Y., Xing, L.-S., Jiang, H., Hu, S.-N., Raikhel, A.S., *et al.* (2015) A Critical Role for CLSP2 in the Modulation of Antifungal Immune Response in Mosquitoes. *PLOS Pathogens*, **11**, e1004931.

Watts, D.M., Thompson, W.H., Yuill, T.M., DeFoliart, G.R. & Hanson, R.P. (1974) Overwintering of La Crosse Virus in Aedes Triseriatus *. *The American Journal of Tropical Medicine and Hygiene*, **23**, 694–700.

Weaver, S.C., Costa, F., Garcia-Blanco, M.A., Ko, A.I., Ribeiro, G.S., Saade, G., *et al.* (2016) Zika virus: History, emergence, biology, and prospects for control. *Antiviral Research*, **130**, 69–80.

Weaver, S.C. & Lecuit, M. (2015) Chikungunya Virus and the Global Spread of a Mosquito-Borne Disease. *New England Journal of Medicine*, **372**, 1231–1239.

Weger-Lucarelli, J., Rückert, C., Chotiwan, N., Nguyen, C., Garcia Luna, S.M., Fauver, J.R., *et al.* (2016) Vector Competence of American Mosquitoes for Three Strains of Zika Virus. *PLOS Neglected Tropical Diseases*, **10**, e0005101.

Weiss, B.L., Wang, J., Maltz, M.A., Wu, Y. & Aksoy, S. (2013) Trypanosome Infection Establishment in the Tsetse Fly Gut Is Influenced by Microbiome-Regulated Host Immune Barriers. *PLOS Pathogens*, **9**, e1003318.

Wen, W., He, Z., Jing, Q., Hu, Y., Lin, C., Zhou, R., *et al.* (2015) Cellular microRNA-miR-548g-3p modulates the replication of dengue virus. *Journal of Infection*, **70**, 631–640.

Werren, J.H., Baldo, L. & Clark, M.E. (2008) Wolbachia: master manipulators of invertebrate biology. *Nature Reviews Microbiology*, **6**, 741–751.

Whitten, M.M.A., Facey, P.D., Del Sol, R., Fernández-Martínez, L.T., Evans, M.C., Mitchell, J.J., *et al.* (2016) Symbiont-mediated RNA interference in insects. *Proceedings of the Royal Society B: Biological Sciences*, **283**, 20160042.

Whyard, S., Erdelyan, C., Partridge, A.L., Singh, A.D., Beebe, N.W. & Capina, R. (2015) Silencing the buzz: a new approach to population suppression of mosquitoes by feeding larvae double-stranded RNAs. *Parasites & Vectors*, **8**, 96.

Wilke, A.B.B. & Marrelli, M.T. (2015) Paratransgenesis: a promising new strategy for mosquito vector control. *Parasites & Vectors*, **8**.

Wilson, A.J. & Harrup, L.E. (2018) Reproducibility and relevance in insect-arbovirus infection studies. *Current Opinion in Insect Science*, **28**, 105–112.

Wu, P., Sun, P., Nie, K., Zhu, Y., Shi, M., Xiao, C., *et al.* (2018) A Gut Commensal Bacterium Promotes Mosquito Permissiveness to Arboviruses. *Cell Host & Microbe*.

Wu, Y., Arumugam, K., Tay, M.Q.X., Seshan, H., Mohanty, A. & Cao, B. (2015) Comparative genome analysis reveals genetic adaptation to versatile environmental conditions and importance of biofilm lifestyle in Comamonas testosteroni. *Applied Microbiology and Biotechnology*, **99**, 3519–3532.

Wykes, G.R. (1952) An investigation of the sugars present in the nectar of flowers of various species. *New Phytologist*, **51**, 210–215.

Xi, Z., Ramirez, J.L. & Dimopoulos, G. (2008) The Aedes aegypti toll pathway controls dengue virus infection. *PLoS Pathogens*, **4**, e1000098.

Xia, H., Hu, C., Zhang, D., Tang, S., Zhang, Z., Kou, Z., *et al.* (2015) Metagenomic Profile of the Viral Communities in Rhipicephalus spp. Ticks from Yunnan, China. *PLOS ONE*, **10**, e0121609.

Xia, Y. & Zwiebel, L.J. (2006) Identification and characterization of an odorant receptor from the West Nile virus mosquito, Culex quinquefasciatus. *Insect Biochemistry and Molecular Biology*, **36**, 169–176.

Xie, P., Xie, Y., Zhang, X., Huang, H., He, L., Wang, X., *et al.* (2013) Inhibition of Dengue Virus 2 Replication by Artificial MicroRNAs Targeting the Conserved Regions. *Nucleic Acid Therapeutics*, **23**, 244–252.

Yadav, K.K., Datta, S., Naglot, A., Bora, A., Hmuaka, V., Bhagyawant, S., *et al.* (2016) Diversity of Cultivable Midgut Microbiota at Different Stages of the Asian Tiger Mosquito, Aedes albopictus from Tezpur, India. *PLOS ONE*, **11**, e0167409.

Yan, H., Zhou, Y., Liu, Y., Deng, Y., Puthiyakunnon, S. & Chen, X. (2014) miR-252 of the Asian tiger mosquito Aedes albopictus regulates dengue virus replication by suppressing the expression of the dengue virus envelope protein. *Journal of Medical Virology*, **86**, 1428–1436.

Yang, J.H., Bhargava, P., McCloskey, D., Mao, N., Palsson, B.O. & Collins, J.J. (2017a) Antibiotic-Induced Changes to the Host Metabolic Environment Inhibit Drug Efficacy and Alter Immune Function. *Cell Host & Microbe*, **22**, 757-765.e3.

Yang, Y., Shan, C., Zou, J., Muruato, A.E., Bruno, D.N., Almeida Medeiros Daniele, B. de, *et al.* (2017b) A cDNA Clone-Launched Platform for High-Yield Production of Inactivated Zika Vaccine. *EBioMedicine*, **17**, 145–156.

Zélé F., Nicot A., Berthomieu A., Weill M., Duron O. & Rivero A. (2014) Wolbachia increases susceptibility to Plasmodium infection in a natural system. *Proceedings of the Royal Society B: Biological Sciences*, **281**, 20132837.

Zélé, F., Nicot, A., Duron, O. & Rivero, A. (2012) Infection with Wolbachia protects mosquitoes against Plasmodium-induced mortality in a natural system. *Journal of Evolutionary Biology*, **25**, 1243–1252.

Zhang, G., Hussain, M. & Asgari, S. (2014) Regulation of arginine methyltransferase 3 by a Wolbachia-induced microRNA in Aedes aegypti and its effect on Wolbachia and dengue virus replication. *Insect Biochemistry and Molecular Biology*, **53**, 81–88.

Zhang, Y., Zhao, B., Roy, S., Saha, T.T., Kokoza, V.A., Li, M., *et al.* (2016) microRNA-309 targets the Homeobox gene SIX4 and controls ovarian development in the mosquito Aedes aegypti. *Proceedings of the National Academy of Sciences*, **113**, E4828–E4836.

Zheng, H., Nishida, A., Kwong, W.K., Koch, H., Engel, P., Steele, M.I., *et al.* (2016) Metabolism of Toxic Sugars by Strains of the Bee Gut Symbiont *Gilliamella apicola*. *mBio*, **7**.

Zhou, Y., Liu, Y., Yan, H., Li, Y., Zhang, H., Xu, J., *et al.* (2014) miR-281, an abundant midgut-specific miRNA of the vector mosquito Aedes albopictus enhances dengue virus replication. *Parasites & Vectors*, **7**, 488.

Zink, S., Van Slyke, G., Palumbo, M., Kramer, L. & Ciota, A. (2015) Exposure to West Nile Virus Increases Bacterial Diversity and Immune Gene Expression in Culex pipiens. *Viruses*, **7**, 5619–5631.

Zouache, K., Michelland, R.J., Failloux, A.-B., Grundmann, G.L. & Mavingui, P. (2012) Chikungunya virus impacts the diversity of symbiotic bacteria in mosquito vector: VIRUS AND BACTERIAL DYNAMICS IN MOSQUITO. *Molecular Ecology*, **21**, 2297–2309.

Zouache, K., Raharimalala, F.N., Raquin, V., Tran-Van, V., Raveloson, L.H.R., Ravelonandro, P., *et al.* (2011) Bacterial diversity of field-caught mosquitoes, Aedes albopictus and Aedes aegypti, from different geographic regions of Madagascar: Bacterial communities of wild Aedes mosquito vectors. *FEMS Microbiology Ecology*, **75**, 377–389.

Vita

Miguel Arturo Saldaña was born on May 23, 1983 in Orem, Utah to Sylvia Jean and Miguel Angel Saldaña. He earned an Associate's Degree in Biology form Utah Valley State College (now Utah Valley Univ.). Miguel completed his Bachelor's Degree in Biology with a minor in Spanish Translation at the University of Texas at Brownsville (now UTRGV). After graduating Miguel was accepted into Baylor College of Medicine's Post Baccalaureate PREP program. He then spent a year working at Harris County's Institute of Forensic Sciences as a Serologist. He then moved on to MD Anderson Cancer Center where he worked in a lab studying Group A Strep bacteria.

Education:

2006 – A.S., Biology, Utah Valley State College, Orem, UT

2009 – B.S., Biology, University of Texas at Brownsville, Brownsville, TX

2019 – Ph.D., Microbiology and Immunology, University of Texas Medical Branch, Galveston, TX

Publications:

1. Bhadra S, **Saldaña MA**, Han HG, Hughes GL, Ellington AD. Simultaneous Detection of Different Zika Virus Lineages via Molecular Computation in a Point-of-Care Assay. Viruses. 2018 Dec 14;10(12). pii: E714. doi: 10.3390/v10120714. PMID: 30558136

2. Hegde S, Khanipov K, Albayrak L, Golovko G, Pimenova M, **Saldaña MA**, Rojas MM, Hornett EA, Motl GC, Fredregill CL, Dennett JA, Debboun M, Fofanov Y, Hughes GL. Microbiome Interaction Networks and Community Structure From Laboratory-Reared and Field-Collected Aedes aegypti, Aedes albopictus, and Culex quinquefasciatus Mosquito Vectors. Front. Microbiol. 2018 Sep 10;9:2160. eCollection 2018. PMID: 30250462

3. Bhadra S, Riedel TE, **Saldaña MA**, Hegde S, Hughes GL, and Ellington AD. Direct nucleic acid analysis of mosquitoes for high fidelity species identification

and detection of *Wolbachia* using a cellphone. PLoS Negl Trop Dis. 2018 Aug 30;12(8):e0006671. eCollection 2018 Aug. PMID: 30161131

4. Ly P, Aizenberg A, Martin T, Lopez M, **Saldaña MA,** Hughes GL, Cabada MM. Intestinal Myiasis Caused by Sarcophaga spp. in Cusco, Peru: A Case Report and Review of the Literature. Case Rep Infect Dis. 2018 May 27;2018:3685439. PMID: 29977632

5. Etebari K, Hegde S, **Saldaña MA**, Widen SG, Wood TG, Asgari S, Hughes GL. Global transcriptome analysis of *Aedes aegypti* mosquitoes in response to Zika virus infection. mSphere. 2017 Nov 22;2(6). pii: e00456-17. eCollection 2017 Nov-Dec. PMID: 29202041.

6. **Saldaña MA**, Etebari K, Hart CE, Widen SG, Wood TG, Thangamani S, Asgari S, Hughes GL. Zika virus alters the microRNA expression profile and elicits an RNAi response in *Aedes aegypti* mosquitoes. PLoS Negl Trop Dis. 2017 Jul 17;11(7):e0005760. eCollection 2017 Jul. PMID: 28715413.

7. **Saldaña MA**, Hegde S, Hughes GL. Microbial Control of Arthropod-Borne Disease. Mem Inst Oswaldo Cruz. 2017 Feb; 112(2):81-93. PMID: 28177042.

8. DebRoy S, **Saldaña M**, Travisany D, Montano A, Galloway-Peña J, Horstmann N, Yao H, González M, Maass A, Latorre M, Shelburne SA. A Multi-Serotype Approach Clarifies the Catabolite Control Protein A Regulon inthe Major Human Pathogen Group A Streptococcus. Sci Rep. 2016 Sep 1;6:32442. PMID: 27580596.

9. Flores AR, Galloway-Peña J, Sahasrabhojane P, **Saldaña M**, Yao H, Su X, Ajami NJ, Holder ME, Petrosino JF, Thompson E, Margarit Y Ros I, Rosini R, Grandi G, Horstmann N, Teatero S, McGeer A, Fittipaldi N, Rappuoli R, Baker CJ, Shelburne SA. Sequence type 1 group B Streptococcus, an emerging cause of invasive disease in adults, evolves by small genetic changes. Proc Natl Acad Sci U S A. 2015 May 19; 112(20):6431-6. Epub 2015 May 4. PMID: 25941374.

10. Horstmann N, Sahasrabhojane P, **Saldaña M**, Ajami NJ, Flores AR, Sumby P, Liu CG, Yao H, Su X, Thompson E, Shelburne SA. Characterization of the effect of the histidine kinase CovS on response regulator phosphorylation in group A Streptococcus. Infect Immun. 2015 Mar; 83(3):1068-77. PMID: 25561708.

11. Horstmann N, **Saldaña M**, Sahasrabhojane P, Yao H, Su X, Thompson E, Koller A, and Shelburne SA. Duel-Site Phosporylation of the Control of Virulence Regulator Impacts Group A Streptococcal Global Gene Expression and Pathogenesis. PLoS Pathog. 10(5):e1004088, 5/1/2014. PMID: 24788524.

12. Shelburne SA, Sahasrabhojane P, **Saldana M**, Yao H, Su S, Horstmann N, Thompson E, and Flores AR. Identification and Characterization of

Streptococcus mitis Strains Causing Severe Clinical Disease in Cancer Patients. Emerg Infect Dis. 20(5):762-71, 5/2014. PMID: 24750901.

13. Sahasrabhojane P, Galloway-Peña J, Velazquez L, **Saldaña M**, Horstmann N, Tarrand J, and Shelburne SA. Species Level Assessment of the Molecular Basis of Fluoroquinolone Resistance Among Viridans Group Streptococci Causing Bacteremia in Cancer Patients. Int J Antimicrob Agents. pii: S0924-8579(14)00067-3, 3/27/2014. PMID: 24721231.

14. Flores AR, Sahasrabhojane P, **Saldaña M**, Galloway-Peña J, Olsen RJ, Musser JM, and Shelburne SA. Molecular Characterization of an Invasive Phenotype of Group A *Streptococcus* Arising During Human Infection Using Whole Genome Sequencing of Multiple Isolates From the Same Patient. J Infect Dis. 209(10):1520-3, 5/2014. Epub 12/4/2013. PMID: 24307742.

Permanent address: 4303 Sugar Bars Dr., Friendswood, TX 77546

This dissertation was typed by Miguel Arturo Saldaña.