GUT MICROBIAL DIVERSITY AND FUNCTION IN MOSQUITOES

by

KERRI LYNN COON

(Under the Direction of Michael R. Strand)

ABSTRACT

Mosquitoes are well recognized as the most important arthropod vectors of pathogens that cause disease in humans and other vertebrates. A long-term research goal of high priority in the biomedical health sciences therefore is to identify novel strategies for mosquito control. The most significant finding of my doctoral studies is that the gut microbiota of *Aedes aegypti* (the yellow fever mosquito) and other mosquito species are essential for development. The mosquito gut harbors a low diversity microbial community comprised primarily of aerobic bacteria. However, the role these bacteria play in mosquito biology is largely unknown. My results demonstrate that the gut microbiota of laboratory-reared *Ae. aegypti* assemble into distinct communities throughout development. My results further show that axenic (bacteria-free) *Ae. aegypti* larvae fail to develop beyond the first instar. However, recolonization of the gut by several gut community members and the non-community member *Escherichia coli*

rescues development of axenic larvae into adults. Comparative studies in other mosquito species from both the laboratory and field suggest a fundamental, but previously unrecognized, dependence by most if not all mosquitoes on their gut microbiota for development. Recent studies have taken advantage of *E. coli* as a model bacterium to identify the mechanisms underlying the function of gut bacteria in mosquito development and their impact on mosquito physiology. Results from a genome-wide functional screen in *E. coli* reveal roles for bacteria in regulating the endocrine and nutritional events leading to molting. Preliminary studies on the contribution of specific gut community members to mosquito reproduction further support a role for gut microbiota in nutrient acquisition and egg production by certain species. Together, these studies provide information on the critical roles gut microbes play in mosquito biology. They also provide crucial information for manipulating mosquito development as a strategy for vector control.

INDEX WORDS: insects, evolution, microbiota, mosquito, development, reproduction, symbiosis

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Dedication

To mom and dad, for all of your support and encouragement.

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CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

Insects are the most abundant and diverse group of animals on Earth (Basset et al. 2012), with more than 1.2 million species known and an estimated 10 million yet to be described (Kikuchi 2009). The evolutionary success of insects has been due in part to their equally diverse associations with beneficial microbes, which have been shown to aid in nutrient acquisition, digestion, defense against natural enemies, growth and reproduction (Bourtzis & Miller 2003; Ferrari & Favre 2011; Engel & Moran 2013).

Many of the most well characterized examples of insect-microbe symbioses involve highly specialized associations that are obligate for both partners (Bourtzis & Miller 2003; Ferrari & Favre 2011; Engel & Moran 2013). Many of these associations

have a nutritional basis—often times consisting of only one or several keystone bacterial species that are essential for host survival and reproduction (Munson et al. 1991; Schroder et al. 1996; Douglas 1998; Chen et al. 1999; Thao et al. 2000; Lo et al. 2003; Degnan et al. 2004; Lefevre et al. 2004; Thao & Baumann 2004; Baumann 2005; Baumann & Baumann 2005; Feldhaar et al. 2007). This is because many insects have evolved to exploit unusual or nutritionally restricted food sources (e.g. plant sap, wood) that lack essential nutrients required for growth and development, including certain amino acids, vitamins, and sterols. These insects therefore rely on symbiotic bacteria to supply the essential nutrients deficient in their diet (Buchner 1965). Several hallmark features are associated with such obligate nutritional symbioses, including strict vertical transmission and sequestration of symbionts inside host cells (Bourtzis & Miller 2003; Ferrari & Favre 2011; Engel & Moran 2013). This host fidelity drives coevolution of symbionts with their hosts and results in mutually dependent relationships, where symbionts are no longer able to survive outside of the host cell environment (Moran et al. 2008; McCutcheon & Moran 2010; McCutcheon & Moran 2012; Wilson & Duncan 2015).

Many insect species also harbor communities of microbes in their digestive tract, which are collectively referred to as the 'gut microbiota' (Engel & Moran 2013). Unlike most obligate symbionts, these communities are often characterized by highly variable

assemblages of bacteria that are acquired from the environment each generation (Broderick et al. 2004; Robinson et al. 2010; Chandler et al. 2011; Priya et al. 2012; Tang et al. 2012). While many studies have characterized taxonomic diversity of gut microbiota across different insect species, functional studies are largely restricted to only a few host taxa. These studies collectively demonstrate that gut microbiota play critical roles in the immunity, metabolism, growth and behavior of their insect hosts (Buchon et al. 2013; Crotti et al. 2013; Engel & Moran 2013; Lee & Brey 2013; Dobson et al. 2015; Thibert et al. 2016), but also suggest that these associations are rarely obligate. Many gut bacterial species can be cultured outside of their hosts, and in some cases axenic insects which lack a gut microbiota can be reared on nutritionally complete diets in the laboratory with minimal effects on growth or development (Bakula 1969).

Mosquitoes are insects of interest because several species vector pathogens as adults that cause disease in humans and other vertebrates. Mosquito larvae hatch from eggs deposited on or near the surface of standing water. Larvae molt through four aquatic instars before pupating and emerging as adults (Clements 1992). Larvae feed on detritus, microorganisms, and small invertebrates in their aquatic habitat (Merritt et al. 1992), while adults of both sexes are terrestrial and feed on sugar sources (Foster 1995). Adult female mosquitoes also must usually feed on blood from a vertebrate host to produce eggs, which is how mosquitoes acquire and transmit disease-causing organisms

(Briegel 2003). Culture-dependent and culture-independent approaches have been used to isolate and identify members of the gut microbiota from several mosquito species collected in the laboratory or field (DeMaio et al. 1996; Lindh et al. 2005; Ponnusamy et al. 2008; Dong et al. 2009; Rani et al. 2009; Chouaia et al. 2010; Gusmao et al. 2010; Cirimotich et al. 2011; Djadid et al. 2011; Wang et al. 2011; Zouache et al. 2011; Boissiere et al. 2012; Chavshin et al. 2012; Osei-Poku et al. 2012; Terenius et al. 2012; Dada et al. 2014; Gimmonneau et al. 2014; Minard et al. 2014; Duguma et al. 2015; Yadav et al. 2015; Buck et al. 2016; Muturi et al. 2016). Most of these studies have focused on adults because of their ability to transmit disease-causing pathogens to humans. These taxonomic surveys indicate that adult mosquitoes contain low diversity bacterial communities, which vary tremendously across species, among individuals, and as a function of collection site. Functional studies in human disease vectors including *Aedes aegypti* (yellow fever mosquito) and *Anopheles gambiae* (African malaria mosquito) also indicate that the gut microbiota can reduce susceptibility of mosquitoes to infection by human pathogens, either through activation of mosquito immune responses, resource competition, or the production of anti-pathogen molecules (Pumpuni et al. 1993; Pumpuni et al. 1996; Gonzalez-Ceron et al. 2003; Xi et al. 2008; Dong et al. 2009; Cirimotich et al. 2011; Joyce et al. 2011; Boissiere et al. 2012; Ramirez et al. 2012; Eappen et al. 2013; Bahia et al. 2014; Ramirez et al. 2014; Gendrin et al. 2015).

Far less attention has been placed on understanding the role of gut microbes in basic mosquito physiology, including the processes regulating larval growth, development, and adult reproduction. Mosquito larvae obtain their gut bacterial community from the aquatic environment they live in (Ponnusamy et al. 2008; Wang et al. 2011; Dada et al. 2014; Gimmonneau et al. 2014). A number of studies have shown that the bacterial composition of the larval aquatic environment determines the composition of the endogenous bacterial flora present in the midgut of adult mosquitoes (Wang et al. 2011; Boissiere et al. 2012, Gimmonneau et al. 2014; Chavshin et al. 2015; Duguma et al. 2015). However, the processes underlying this transition are not well understood, and the degree to which the level of bacterial richness impacts host physiology is not known. Chouaia et al. (2012) observed that *An. stephensi* larvae fed rifampicin exhibit delays in growth that are rescued by adding antibiotic-resistant *Asaia* to rearing pans. Feeding antibiotics to adult *Ae. aegypti* also reduces the abundance of bacteria in the midgut after a blood meal, which correlates with reduced hemolytic activity and egg production (Gaio et al. 2011). While these studies suggest the microbial community is important in regulating mosquito growth and reproduction, it remains unclear how specific members of the community contribute to normal mosquito development or egg production. These studies also relied on antibiotics, which do not fully eliminate bacteria from the gut and introduce uncontrolled alterations in the

composition of the community that confound interpretation of functional assays. Understanding the function of gut bacterial communities or specific community members therefore requires methods to produce axenic (i.e. germ-free) and gnotobiotic animals, which are colonized by one or more known bacterial species. Identifying bacterial gene product(s) associated with development and reproductive success of mosquito vector species has important implications for the identification of novel molecules or methods for reducing mosquito survival and pathogen transmission in the field.

The primary goal of this dissertation was to understand the function of gut bacteria in mosquito development and reproduction. This work has integrated many approaches, from experimental manipulation of gut bacterial communities in different mosquito species from both the laboratory and field to genomic and functional analyses of specific bacterial community members. My results are outlined as follows:

In Chapter 2, I characterized the bacteria present in several mosquito species reared in the laboratory by pyrosequencing. I also reported methods for producing axenic mosquitoes, which allowed me to manipulate and selectively colonize the larval or adult gut in a controlled fashion. Using these methods, I showed that mosquitoes require living bacteria in their gut to develop. I also identified several bacterial species, including the non-community member *Escherichia coli,* that colonize the larval gut and

restore development into adults. These studies were significant because they stepped beyond characterization of taxonomic diversity to provide novel insights into the function of gut microbiota in mosquitoes.

In Chapter 3, I completed field studies to examine how gut bacterial communities in field mosquitoes compare to lab populations, and how communities vary within and between different mosquito species and their larval habitats. Illumina sequencing revealed substantial variation between communities in larvae collected from different sites, while communities in larvae from the same site were similar. Field-derived mosquitoes also failed to develop in the absence of bacteria but were rescued by living bacteria. The results of these studies were significant because they showed that despite variation in gut microbiota, all mosquitoes rely on a conserved factor provided by bacteria.

In Chapter 4, I screened a collection of single-gene deletion mutants for 3,985 non-essential genes in *E. coli*. I identified a subset of mutants defective in rescuing mosquito development, and was able to fully restore larval growth by transforming these mutants with an expression plasmid carrying the defective gene. Subsequent work demonstrated that these genes intersect with pathways known to regulate growth in insects—in particular the release of ecdysteroid hormones, which stimulate molting. These results are of translational significance because the processes involved may be

amenable to disruption, which could be used as a tool for controlling species that transmit human diseases.

In Chapter 5, I examined the role of gut bacteria in egg production by *Ae. aegypti* and *Ae. atropalpus* mosquitoes, which are closely related but differ in life history in that *Ae. aegypti* females are anautogenous and must blood feed to produce eggs, while *Ae. atropalpus* females are facultatively autogenous and produce a first clutch of eggs without blood feeding. Results implicated specific gut bacterial species in nutrient acquisition and egg production by facultatively autogenous *Ae. atropalpus*. In contrast, composition of the gut microbiota did not affect egg production by *Ae. aegypti,* which obtain nutrients by blood feeding. This was the first study to assess the contribution of individual gut community members to mosquito reproduction. The results of this study also established the gut microbiota as a previously unrecognized factor in the evolution of autogeny in mosquitoes.

Ultimately, this dissertation provides new insights into the gut microbiota and its function in mosquitoes. Further, this work establishes a foundation for studying the molecular mechanisms underlying mosquito-bacteria interactions and their potential as targets for vector control.

CHAPTER 2

MOSQUITOES RELY ON THEIR GUT MICROBIOTA FOR DEVELOPMENT[1]

[1]Coon, K.L., Vogel, K.J., Brown, M.R. and M.R. Strand. 2014. *Molecular Ecology* 23:2727-2739.

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2.1 Abstract

Field studies indicate adult mosquitoes (Culicidae) host low diversity communities of bacteria that vary greatly among individuals and species. In contrast, it remains unclear how adult mosquitoes acquire their microbiome, what influences community structure, and whether the microbiome is important for survival. Here, we used pyrosequencing of 16S rRNA to characterize the bacterial communities of three mosquito species reared under identical conditions. Two of these species, *Aedes aegypti* and *Anopheles gambiae*, are anautogenous and must blood-feed to produce eggs, while one, *Georgecraigius atropalpus*, is autogenous and produces eggs without blood feeding. Each mosquito species contained a low diversity community comprised primarily of aerobic bacteria acquired from the aquatic habitat in which larvae developed. Our results suggested that the communities in *Ae. aegypti* and *An. gambiae* larvae share more similarities with one another than with *G. atropalpus*. Studies with *Ae. aegypti* also strongly suggested that adults transstadially acquired several members of the larval bacterial community, but only four genera of bacteria present in blood fed females were detected on eggs. Functional assays showed that axenic larvae of each species failed to develop beyond the first instar. Experiments with *Ae. aegypti* indicated several members of the microbial community and *Escherichia coli* successfully colonized axenic larvae and rescued development. Overall, our results provide new insights about the acquisition and

structure of bacterial communities in mosquitoes. They also indicate that three mosquito species spanning the breadth of the Culicidae depend on their gut microbiome for development.

Keywords: bacteria, development, evolution, insects, microbial biology

2.2 Introduction

Microbes are well recognized to play important roles in the biology of metazoans. In insects, associations ranging from obligate intracellular symbionts to environmentally acquired gut microbiota have been studied in several taxa (Moran 2006; Engel & Moran 2013; Lee & Brey 2013). Mosquitoes (Culicidae) are of significant interest because several species vector disease-causing organisms to humans and other vertebrates. Larval-stage mosquitoes are aquatic and feed on detritus, microorganisms and invertebrates (Clements 1992; Merritt et al. 1992), while adults of both sexes feed on sugar sources (Foster 1995). Adult females of most species also must feed on blood from a vertebrate host to produce eggs, which can result in the acquisition and transmission of pathogens (Clements 1992; Briegel 2003).

It has long been known that bacteria inhabit the mosquito gut (Chao et al. 1963), and in recent years, community members from several species have been identified by culture-dependent (DeMaio et al. 1996; Dong et al. 2009; Chouaia et al.

2010; Gusmão et al. 2010; Cirimotich et al. 2011; Djadid et al. 2011; Oliveira et al. 2011; Terenius et al. 2012) and culture-independent approaches (Wang et al. 2011; Boissiere et al. 2012; Chavshin et al. 2012; Osei-Poku et al. 2012). Most of these studies have focused on adults because of results showing gut microbes affect susceptibility to infection by pathogens mosquitoes transmit to humans (Cirimotich et al. 2011; Wang et al. 2011; Boissiere et al. 2012). Culture-independent data sets overall indicate that adult mosquitoes contain low diversity bacterial communities, which vary between individuals and species (Boissiere et al. 2012; Osei-Poku et al. 2012). Functional data suggest the gut microbiome can reduce susceptibility to infection by human pathogens through modulation of the mosquito immune response or production of antiparasite molecules by certain community members (Xi et al. 2008; Dong et al. 2009; Cirimotich et al. 2011; Bahia et al. 2014).

The high variation in bacterial taxa detected in field-collected adults strongly suggests a dominant role for the environment in determining community composition (Boissiere et al. 2012; Osei-Poku et al. 2012). However, it remains uncertain whether adult mosquitoes predominantly acquire bacteria transstadially from larvae or through their own feeding activity (Pumpuni et al. 1996; Moll et al. 2001; Briones et al. 2008; Lindh et al. 2008). The composition of gut bacterial communities can also be strongly influenced by diet, which in the case of mosquitoes differs with life stage and can vary

between species (Merritt et al. 1992). Lastly, antibiotic treatment of larvae slows growth of *Anopheles* spp. (Wotton et al. 1997; Chouaia et al. 2012), but the role of the gut microbiome in this response is largely unclear.

Given this literature, the first question addressed in this study was whether resident bacterial communities are similar or different between mosquito species when reared identically in the laboratory. The Culicidae is monophyletic and consists of two subfamilies, the Anophilinae and Culicinae, which diverged 145–200 Mya (Reidenbach et al. 2009). We therefore selected one culicine, *Aedes aegypti* (UGAL strain), and anopheline, *Anopheles gambiae* (G3 strain), for study, which must blood-feed on a vertebrate host as adults to produce eggs (i.e. anautogenous). We also selected a second culicine for study, *Georgecraigius atropalpus* (Rockpool strain), which is closely related to *Ae. aegypti* but fundamentally differs in life history because females emerge as adults and produce a first clutch of eggs without blood feeding (facultatively autogenous) (Gulia-Nuss et al. 2012). The second question we addressed was whether the gut community of mosquitoes is important for development. This was addressed by producing axenic larvae devoid of any living bacteria and gnotobiotic larvae that were colonized by a single bacterial species. Overall, our results showed that each mosquito species contains a simple bacterial community with *An. gambiae* and *Ae. aegypti* being more similar to one another than to *G. atropalpus*. Our results also showed that axenic

larvae cannot develop, but several community members and *Escherichia coli* rescued development.

2.3 Materials and Methods

Conventionally reared mosquitoes

Aedes aegypti, *Anopheles gambiae* and *Georgecraigius atropalpus* were conventionally reared in the same insectary at 28 °C, ~60% relative humidity (RH) and 16 h light:8 h dark photoperiod (Riehle & Brown 2002). Larvae were fed a standard diet consisting of finely ground rat chow (Purina)/lactalbumin/brewers yeast (1:1:1) in open aluminium rearing pans containing distilled water produced in the laboratory. Pupae in water from the larval rearing pans were transferred to plastic cages where adults emerged and commonly imbibed water from rearing pans. After emergence, conventionally reared adults were provided 10% sucrose in water *ad libitum*. *G. atropalpus* females thereafter laid a first clutch of eggs on filter paper. Adult female *Ae. aegypti* and *An. gambiae* were blood fed (BF) 2 days postemergence on an anesthetized rat until engorged. Females then laid a clutch of eggs ~36 h later on filter paper. Eggs from each species were stored in humidified containers at room temperature until needed.

DNA extraction

Total genomic DNA was isolated from water in rearing pans containing conventionally reared mosquito larvae of each species that had moulted to the final (fourth) instar (Figure 2.1). DNA was also isolated from 40 fourth instars from the same rearing pans (Figure 2.1). Larvae were surface-washed with 70% EtOH to eliminate most bacteria from their cuticle, dried and then homogenized in liquid nitrogen. DNA from the water and larval samples was then isolated using the Gentra Puregene Yeast/Bacteria Kit (Qiagen).

Four additional samples were collected from *Ae. aegypti*: (i) 80 non-blood fed (NBF) adults, which emerged from surface-sterilized pupae, (ii) 40 three-day-old adults, which emerged from surface-sterilized pupae and BF on a surface-sterilized host 24 h previously, (iii) egg masses oviposited onto sterile paper in sterile water by females from surface-sterilized pupae (STR) and (iv) egg masses oviposited by conventionally reared females (ConR) (Figure 2.1). Pupae produced from conventionally reared larvae were surface-sterilized by placing in 2% bleach for 2 min and rinsing 3× in sterile water. These pupae were then placed in sterile water in a sterile plastic chamber and allowed to emerge into adults. The 80 NBF adults were prevented from imbibing water by collecting them immediately after emergence from the pupal cuticle. The 40 BF adults were also collected immediately after emergence, held in sterile cages and provided a

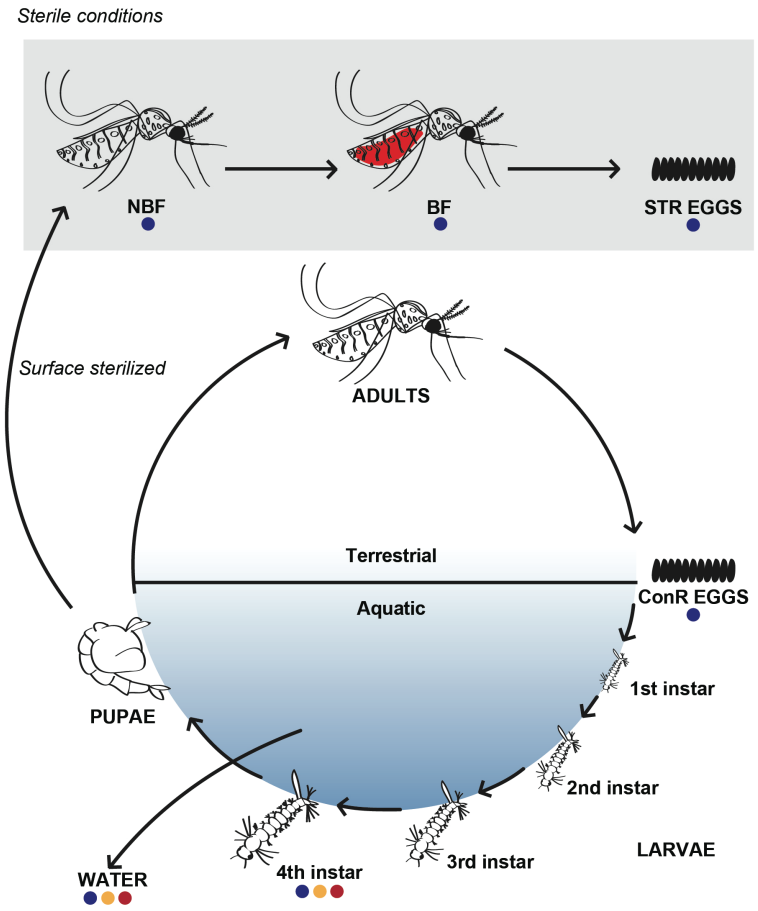


Figure 2.1. Life cycle of *Aedes aegypti*, *Anopheles gambiae* and *Georgecraigius atropalpus* and samples collected for 454 pyrotagging analysis. Each species oviposits eggs that hatch in aquatic habitats where the larval-stage feeds and acquires bacteria that colonize the digestive tract. Larvae undergo metamorphosis after the fourth instar to form pupae that float on the surface of the aquatic habitat. Adults emerge from the pupal stage and persist in terrestrial habitats. Newly emerged adult mosquitoes often imbibe water from the aquatic habitat. Adults of each species also feed on sugar sources. *G. atropalpus* is autogenous and oviposits a first clutch of eggs without taking a bloodmeal. Adult female *Ae. aegypti* and *An. gambiae* are anautogenous and must blood-feed on a vertebrate host to lay eggs. Circles indicate life stages sampled for pyrosequencing for *Ae. aegypti* (blue), *An. gambiae* (yellow) and *G. atropalpus* (red), respectively. For conventionally reared mosquitoes, samples were collected from the water in which larvae developed and from fourth instars of each species. For *Ae. aegypti*, conventionally reared pupae were surface-sterilized to produce non-blood fed (NBF) adults and blood fed (BF) adults after blood feeding on a surface-sterilized host. BF adults then laid STR eggs. Eggs laid by conventionally reared *Ae. aegypti* females were named ConR eggs (see 'Materials and Methods' for additional details).

sterile water source for 2 days before blood feeding. These NBF and BF adults were then surface-rinsed with 70% EtOH followed by homogenization and DNA extraction. STR egg masses and ConR egg masses were not rinsed with EtOH before DNA extraction. All water used for these samples was sterilized by autoclaving. Paper used for egg laying was also autoclaved, while hosts used for blood feeding were surface-sterilized by washing with 70% EtOH.

PCR and pyrosequencing

Bacterial 16S rRNA gene V1-V2 variable regions were PCR-amplified using the universal primers 27F-short (Martinson et al. 2011) and 342R (Kunin et al. 2010) containing multiplex identifier sequences (Table A.1). Amplicons were generated from each sample in four separate 20 μl PCRs that were pooled prior to purification. Each reaction contained iProof High-Fidelity Polymerase (Bio-Rad), the aforementioned primers, dNTP mix and 100 ng of template with the following reaction conditions: denaturation at 98 °C for 30 s, followed by 30 cycles of 98 °C for 10 s, 55 °C for 20 s, and 72 °C for 20 s, and final extension at 72 °C for 5 min. Reactions without template served as a negative control. The resulting products were isolated using the MinElute PCR purification kit (Qiagen). Samples (300 ng) were pooled and submitted to the Georgia Genomics Facility, where pyrosequencing adapters were ligated to the pooled

amplicons. A total of three libraries were constructed and sequenced using Titanium series reagents and the Roche 454 GS-Jr sequencing platform.

Reads were converted to FASTQ format and filtered by the following criteria: forward and reverse primer sequences (spacer, barcode and amplification primer) must perfectly match the synthesized sequence; all bases must have a PHRED equivalent score of 30 or higher (per base error rate of 0.1%); reads must be between 300 and 321 bp in length; and no ambiguous bases (an N) present (Kunin et al. 2010). Reads were then subjected to chimera analysis using Chimera Slayer (Haas et al. 2011). Following quality filtering, reads were de-multiplexed using an in-house Perl script. The resulting data were analysed using the Quantitative Insights Into Microbial Ecology (QIIME) pipeline (Lozupone & Knight 2005; DeSantis et al. 2006; Wang et al. 2007; Caporaso et al. 2010a,b; Edgar 2010; Price et al. 2010; Haas et al. 2011; McDonald et al. 2012) and default parameters. QIIME clustered reads into operational taxonomic units (OTUs) at 97% (species-level) sequence identity to compare OTU abundance between samples. If an OTU contained fewer than five reads, they were omitted from downstream analyses. Chao1 and Shannon diversity indices were calculated for each sample as part of the QIIME analysis.

Isolation of bacterial community members and taxonomic assignment

Aedes aegypti eggs, fourth instars and adults (NBF and BF) from our conventional cultures were rinsed in 70% EtOH, transferred to 100 µl of sterile phosphate buffered saline and homogenized. Larval homogenates were serially diluted (up to 10^{-7}) and plated on Luria Broth (LB), brain–heart infusion (BHI), tryptic soy agar (TSA), or blood agar plates at 28 °C for 24–72 h. Colonies were isolated by restreaking onto fresh plates, followed by DNA isolation using the Gentra Puregene DNA isolation kit. Universal primers were used to amplify ~1465 bp of the 16S gene by PCR with products purified using the QIAquick gel extraction kit (Qiagen), cloned into TOPO TA (Invitrogen) and transformed into *Escherichia coli*. Plasmids were isolated from overnight cultures using the GeneJet miniprep kit (Thermo Scientific) and Sanger-sequenced. Vector trimming and quality analysis of sequence data were performed using Lasergene (DNASTAR, Madison, Wis. USA) followed by assignment of a bacterial taxonomic hierarchy using the Ribosomal Database Project (RDP) Release 10 classifier (Cole et al. 2009). Isolates of interest were stored as glycerol stocks at −80 °C. Sequence data were then used to design genus-specific primers that amplified a portion of the 16S rRNA gene of select community members that were successfully isolated and cultured. In brief, regions of interest were identified by aligning the sequence from a given isolate

with homologous sequences from GenBank using MAFFT (Katoh et al. 2005). Regions of low conservation between isolates and available sequences were then selected for primer design. Specificity of resulting primers (Table A.1) for a given isolate was verified by PCR using DNA from all other bacterial isolates cultured from mosquitoes.

Axenic and gnotobiotic mosquitoes

Axenic (bacteria-free) larvae were produced by placing eggs from each species into sterile Petri dishes containing 70% EtOH for 5 min, transferring to a solution of 3% bleach and 0.1% ROCCAL-D (Pfizer) for 3 min, transferring again to 70% EtOH for 5 min and rinsing three times in sterile water. Ten eggs were placed in 25-cm^2 cell culture flasks (Corning) containing 20 ml of sterile water. First instars hatched ca. 2–12 h later and were fed standard diet or fish food (TetraMin) (2 mg) sterilized by exposure to 5 mGy from a cobalt 60 gamma radiation source housed on the University of Georgia campus (College of Veterinary Medicine). Sterility of larvae and diet were confirmed by culture-based and PCR analysis using universal 16S rRNA primers (Table A.1), which indicated no viable bacteria were present. Gnotobiotic larvae were generated by picking a colony of a given bacterial isolate (Table A.2) and inoculating into 25-cm^2 cell culture flasks (Corning) that contained 20 ml of sterile water, sterilized standard diet (2 mg)

and 7–10 axenic first instars. Cultures of gnotobiotic larvae were reared to adulthood by adding new sterilized standard diet every other day until pupation.

We analysed individual larvae and adults to assess colonization and transmission of bacteria in gnotobiotic mosquitoes. Larvae were rinsed in 70% EtOH, while newly emerged adults were collected from surface-sterilized pupae in 2% bleach as described above. Genomic DNA was then isolated from each individual followed by PCR analysis using universal or genus-specific primers. To assess whether bacteria in some manner condition the medium mosquitoes consume, bacteria were added to sterilized standard diet in water and incubated for 72 h at 28 °C. The food mixture was then centrifuged at 835 x *g* for 5 min followed by filter sterilization to produce bacteria-conditioned water. Axenic larvae were then placed in either: (i) conditioned water with or without new sterilized diet or (ii) conditioned water with sterilized diet plus the bacterium used to condition the water. The proportion of first instars that developed into adults and total development time (days) were determined by inspecting cultures daily. Data were analysed by one-way ANOVA, followed by Dunnett's test. Survival data were arcsin-transformed prior to ANOVA. All statistical analyses were performed using R (http://www.r-project.org/).

Larval feeding behaviour

To monitor feeding by mosquito larvae, dye-based feeding assays were conducted using heat-killed *E. coli* labelled with fluorescein isothiocyanate (FITC) (Beck & Strand 2003) or sterilized diet labelled for 1 h with acridine orange (AO) (200 μg/ml) in water. FITC-labelled bacteria (1×10^3 colony-forming units per μl) were added to wells of a 24-well culture plate (Corning) containing 1 ml of water, 10–20 non-sterile or axenic larvae and standard diet (500 μg). AO-labelled diet was added to cultures containing non-sterile or axenic larvae. Images were taken at 2-h intervals using a Leica DMIRE2 stereoscope fitted with a 488-nm filter, Hamamatsu C4742-95 digital camera and SimplePCI software. Total fluorescence corrected for background was measured using ImageJ with a region-of-interest (ROI) defined around the larva.

2.4 Results

Pyrotagging analysis of mosquito bacterial communities

As previously noted, our first goal was to assess whether different mosquito species host similar bacterial communities when reared identically. To address this question, we incorporated phylogeny and life history in selecting *Aedes aegypti*, *Anopheles gambiae* and *Georgecraigius atropalpus* for study. For each of these species, we examined the

bacteria present in the aquatic habitat (water in rearing pans) and in larvae collected as fourth instars (Figure 2.1). For *Ae. aegypti*, we also analysed the bacteria present in NBF and BF adults collected from surface-sterilized pupae, eggs laid by BF adults from surface-sterilized pupae (STR) and eggs laid by adults from our conventional culture (ConR) (Figure 2.1).

Samples were assigned to three runs followed by pyrosequencing of 16S rRNA gene amplicons. After quality filtering and removal of chimeric sequences, this generated 33,806 (run 1, 63% of total reads), 77,430 (run 2, 60%) and 85,398 reads (run 3, 69%) (Table 2.1). These reads collapsed into 4,282, 9,229 and 6,998 non-redundant reads, which were unevenly distributed among samples (Table 2.1). After demultiplexing, sequences were grouped by percentage similarity to form operational taxonomic units (OTUs) at a cut-off threshold of 97%. OTUs represented by five or fewer reads were further interpreted as likely contaminants and discarded (Kunin et al. 2010). Quality differences between libraries resulted in fewer sequences passing filtering requirements in sequencing of the first run, which resulted in fewer OTUs than detected in runs 2 and 3 (Table 2.1). Rarefaction analyses tended towards saturation, but did not fully plateau for most samples, indicating that some community members were missed in our analyses (Figure A.1). However, the total phylogenetic distance observed in species accumulation curves approached asymptotes, suggesting that OTUs missed in sampling did not

include taxa distantly related from those captured in our data sets (Figure A.1). Taken together, mosquito samples contained fewer OTUs than detected in water, which suggested only a subset of bacteria in the aquatic habitat colonized each species (Table 2.1). The relatively low number of OTUs detected in mosquito samples also indicated that bacterial community diversity was low.

Table 2.1. Sequencing statistics from 454 pyrotagging experiments.

Sample	**Filtered reads (% of total reads)**	**Non-redundant reads**	**OTUs >5 reads**
Run 1	33,806 (62.8)	4,282	--
Aedes aegypti water	12,231	2,535	123
Ae. aegypti larvae	5,131	615	66
Ae. aegypti NBF adults	713	168	35
Ae. aegypti BF adults	15,731	964	22
Run 2	77,430 (60.4)	9,229	--
Georgecraigius atropalpus water	12,986	1,906	144
Anopheles gambiae water	22,781	3,178	161
Ae. aegypti eggs (ConR)*	28,150	2,960	72
Ae. aegypti eggs (STR)*	13,513	1,185	53
Run 3	85,398 (69.2)	6,998	--
G. atropalpus larvae	26,064	2,331	91
An. gambiae larvae	59,334	4,667	161

**Ae. aegypti* ConR eggs were laid by conventionally reared females, while *Ae. aegypti* STR eggs were laid by BF females which emerged from surface-sterilized pupae.

Bacterial communities in *Aedes aegypti* and *Anopheles gambiae* larvae differ from *Georgecraigius atropalpus*

Most sequences identified in water from rearing pans and conventionally reared larvae belonged to five bacterial phyla: Firmicutes, Actinobacteria, Bacteroidetes, Betaproteobacteria and Gammaproteobacteria (Figure 2.2). Water contained sequences

belonging to the Flavobacteriaceae, Comamonadaceae and Microbacteriaceae, while Clostridiaceae were also present in water from the rearing pans containing *An. gambiae* and *G. atropalpus* larvae (Figure 2.2). We detected the same bacterial families in water and larvae, but their relative abundance differed (Figure 2.2). Over 95% of the sequences from *Ae. aegypti* larvae belonged to the Flavobacteriaceae (43%) and Microbacteriaceae (54%), while no other taxa comprised >2% of reads. In *An. gambiae*, 86% of sequences belonged to the Flavobacteriaceae with the balance belonging to the Sphingobacteriaceae, Comamonadaceae, Microbacteriaceae, Pseudomonadaceae and Clostridiaceae. *Georgecraigius atropalpus* larvae in contrast contained a community dominated by Betaproteobacteria belonging to the Procabateriaceae, Neisseriaceae and Comamonadaceae (63%).

Plotting OTU overlap in the water samples showed that some community members were unique, but most were shared between two or more species (Figure 2.3A). In contrast, approximately half of the OTUs in *An. gambiae* and *G. atropalpus* larvae were unique (Figure 2.3B). *Aedes aegypti* larvae had a smaller proportion of unique OTUs (26%) (Figure 2.3B), but this was likely due to shallower sampling (Table 2.1). We also found that a majority of OTUs in larvae overlapped with the water where they developed (Figure 2.3C). We further examined community structure by performing a principal component analysis (PCA) on the Unifrac distances in each sample. Unifrac

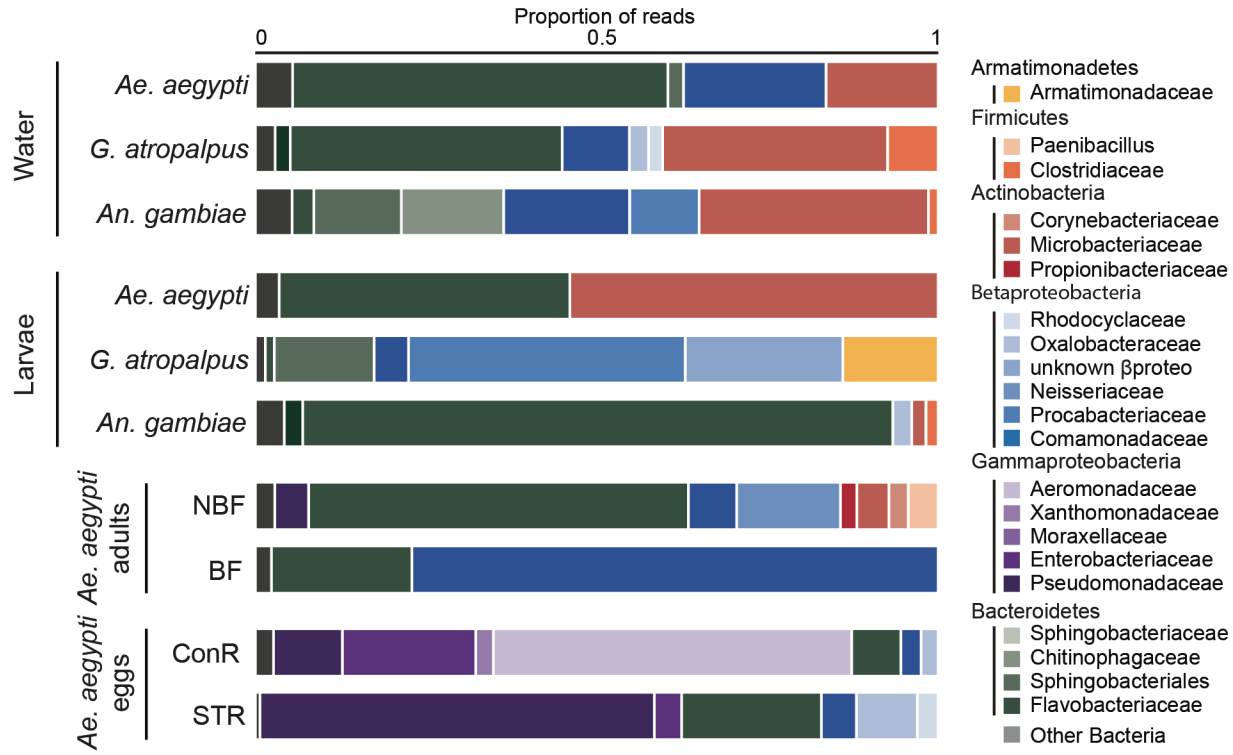


Figure 2.2. Bacterial composition at the phylum and family levels in laboratory-reared *Aedes aegypti*, *Anopheles gambiae* and *Georgecraigius atropalpus*. Water and larval samples (fourth instars) were analysed for each species. Adult and egg samples were analysed for only *Ae. aegypti*. Each bar graph presents the proportion of sequencing reads assigned to a given bacterial family. Only categories >2% are presented. NBF, non-blood fed adult female; BF, adult female 24 h after blood feeding on a host; ConR, eggs laid by conventionally reared *Ae. aegypti* females; STR, eggs laid by females that emerged from surface-sterilized pupae.

uses phylogenetic distances between sequences within samples to assess similarities among samples (Lozupone & Knight 2005; Lozupone et al. 2007). PCA axes 1 and 2 explained 60% of the variation between samples when only the presence or absence of bacterial taxa was considered (unweighted Unifrac distances) (Figure A.2). This increased to 79% when taxon abundance was included (weighted Unifrac distances)

(Figure A.2). The water and larval communities of *Ae. aegypti* clustered with one another in our data sets, but this clustering was lost for *G. atropalpus* and *An. gambiae* using weighted data (Figure A.2). Overall, these findings indicated many of the bacterial taxa in larvae were present in their aquatic habitat, while differences in taxon abundance suggested the water communities associated with each mosquito species were variable.

Bacterial community composition changes during *Aedes aegypti* development

A few bacterial taxa are known to be transstadially transmitted in *Anopheles* spp. (Briones et al. 2008; Lindh et al. 2008; Damiani et al. 2010). However, other data suggest mosquitoes clear most bacteria from the larval gut preceding pupation, which has led to the suggestion that mosquito larvae in general transfer few community members to adults (Moll et al. 2001). It has also been suggested that adults could reacquire bacteria from the larval habitat by imbibing water after eclosing from the pupal stage (Lindh et al. 2008). We assessed whether bacteria in *Ae. aegypti* are transstadially transmitted from the larval gut to adults by surface-sterilizing pupae, transferring them to sterile water and then preventing the resulting adults from imbibing any of the water where the pupae were held. NBF adults were then processed

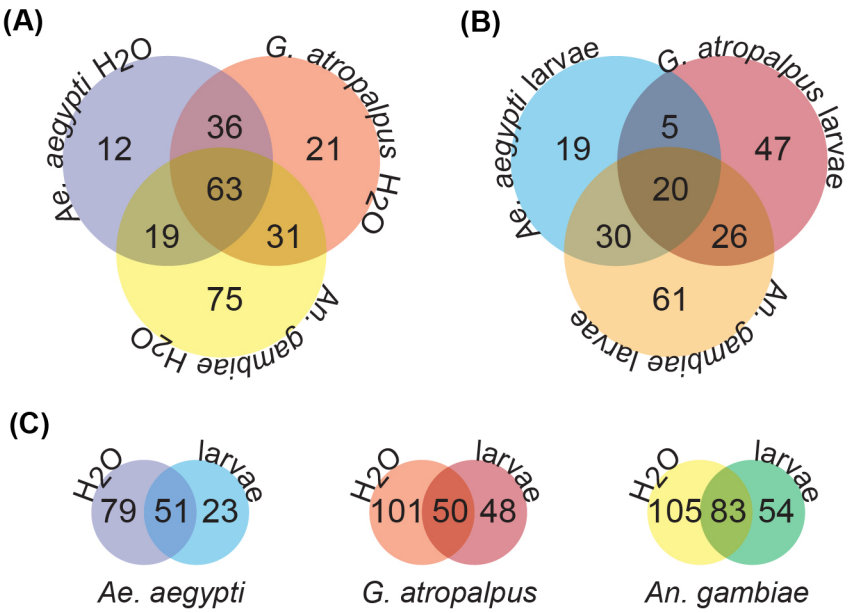


Figure 2.3. Overlap between OTUs in (A) the water habitat larvae of each mosquito species were reared in, (B) the larval stage of each species and (C) water and larvae for each species.

immediately after emergence, while BF adults were held in sterile chambers with only a sterile water source available for consumption before blood feeding on a host. By taking these steps, we minimized the opportunity for any bacteria other than species present in the larval gut to be acquired by adults after emergence. The resulting data indicated that bacterial diversity declined from 74 OTUs in larvae to 39 in NBF adult females and 22 in BF adults (Table 2.1). A few OTUs detected in NBF and BF *Ae. aegypti* adults were absent from larvae, but all belonged to genera present in larvae. Thus, no unique bacteria were detected in adults, which suggested most if not all of the bacteria in our adult samples were acquired transstadially.

In contrast, the most abundant community members in *Ae. aegypti* larvae (Microbacteriaceae) decreased in NBF adults, while a number of flavobacterial and gammaproteobacterial families increased (Figure 2.2). Blood feeding further reduced the abundance of dominant larval community members, while greatly increasing Comamonadaceae (Figure 2.2). The low number of reads from NBF adults resulted in their removal from PCA, although the BF community was included. In both weighted and unweighted analyses, the BF samples were most similar to sterile eggs. The large difference in reads in the BF sample is consistent with other studies showing that certain gut bacteria greatly proliferate following a bloodmeal (Gaio et al. 2011; Oliveira et al. 2011).

Nearly all community members in larval- and adult-stage *Ae. aegypti* were aerobes or facultative anaerobes with only one obligate anaerobe taxon (*Clostridium*) detected. One genus of Flavobacteriaceae (*Chryseobacterium*) was common in all life stages. Two genera of Microbacteriaceae abundant in larvae (*Leucobacter* and *Microbacterium*) were rare in water and nearly absent from adults. Five taxa at low abundance in larvae were abundant in NBF adults (*Pseudomonas*, *Paenibacillus*, *Aeromonas*, *Aquitalea*, *Stenotrophomonas*), while *Chryseobacterium* plus the genus *Delftia* (Comamonadaceae) dominated BF adults with 92% of reads. Other low abundance genera detected in adults included *Enterobacter*. Among all of the OTUs

identified, only five were detected in larvae, NBF adults and BF adults: *Chryseobacterium*, *Delftia*, *Acinetobacter*, *Paenibacillus* and an unclassified member of the Enterobacteriaceae. Although *Wolbachia* is known to infect mosquitoes (Kittayapong et al. 2000), no sequences corresponding to *Wolbachia* were present in our pyrosequencing data sets, while PCR screening using specific primers (Table 2.1) confirmed the absence of *Wolbachia* in our laboratory populations.

Our own results (see below) indicated no bacteria were present in eggs of our *Ae. aegypti*, *An. gambiae* or *G. atropalpus* cultures, but bacteria do reside on the egg surface (Chao et al. 1963). Pyrosequencing showed that egg clutches from conventionally reared *Ae. aegypti* harboured an abundance of Gammaproteobacteria (Pseudomonadaceae, Enterobacteriaceae and Aeromonadaceae) and to a lesser degree Flavobacteriaceae (Figure 2.2A). Eggs laid by females from sterilized pupae also had a high relative abundance of Gammaproteobacteria, primarily Pseudomonadaceae, plus Flavobacteriaceae and Betaproteobacteria (Figure 2.2A). However, only seven OTUs in four genera, *Chryseobacterium*, *Delftia*, *Acinetobacter* and *Stenotrophomonas*, present in BF adults were detected on eggs. This resulted in the bacterial communities on eggs clustering separately in our PCA analyses (Figure A.2). These data also suggested that only a few bacterial taxa in adults are potentially transmitted from the egg surface to larvae of the next generation.

Axenic mosquitoes do not develop

Having characterized the bacterial community in each species, we next asked whether their microbiome was functionally important for development. Although antibiotic treatment greatly reduces bacterial abundance in mosquitoes (Cirimotich et al. 2011; Chouaia et al. 2012), we produced axenic mosquitoes by surface-sterilizing eggs. Hatch rates of sterilized eggs for each species exceeded 90% and did not differ from non-sterilized eggs ($\chi^2 = 1.6$, $P = 0.3$). An abundance of bacteria could be cultured on nutrient or blood agar plates from homogenates of first instars hatched from non-sterilized eggs. In contrast, no bacteria grew on plates using first instars hatched from surface-sterilized eggs. In addition, no amplicons were generated from these larvae using 16S universal primers and total genomic DNA as template. We thus concluded surface sterilization of eggs produced axenic first instars. By the same methods, we confirmed that gamma irradiation sterilized our standard diet and commercial fish food. When axenic *Ae. aegypti*, *An. gambiae* or *G. atropalpus* first instars were placed in sterile water and fed sterilized diet, all died after 5 days without moulting (Figure 2.4). We considered the possibility that diet sterilization compromised nutritional suitability, but two lines of evidence indicated this was not the case. First, axenic larvae fed sterilized diet in flasks containing 20 ml of sterile water plus 100 µl of water from our conventional cultures developed normally as did conventionally reared larvae fed

sterilized diet (Figure 2.4). Second, axenic larvae fed sterilized fish food, which is also commonly used to rear mosquitoes, failed to develop, while non-sterile larvae fed sterilized fish food developed normally.

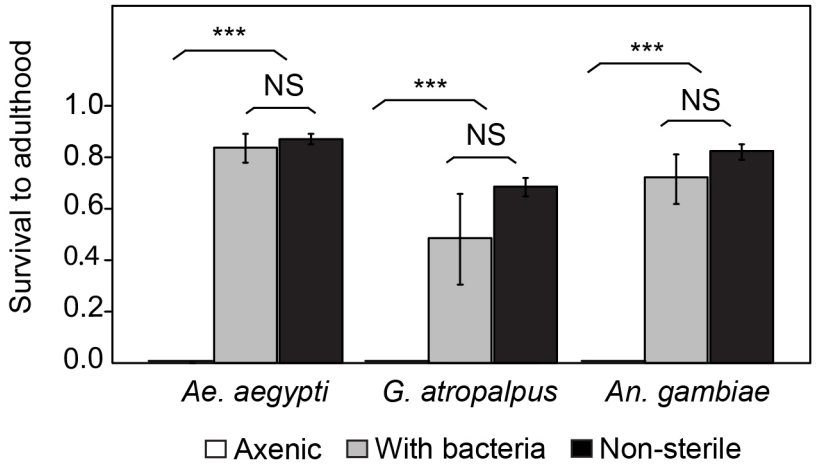


Figure 2.4. Survival of first-instar *Aedes aegypti*, *Anopheles gambiae* and *Georgecraigius atropalpus* to adulthood under three conditions: axenic larvae fed sterilized diet (Axenic); axenic larvae fed sterilized diet plus bacteria from the conventional laboratory habitat (With bacteria), and conventional, non-sterile larvae fed sterilized diet (Non-sterile). A minimum of 50 larvae per treatment was assayed. The proportion of larvae that developed into adults is presented. Columns indicate mean values with 95% confidence intervals. ANOVA analyses were conducted separately for each species with multiple comparisons made by Tukey–Kramer HSD (NS, treatments not significantly different; ***, Axenic treatment significantly different from the With bacteria and Non-sterile treatments, $P < 0.0001$).

Several bacterial community members rescue development

We used *Ae. aegypti* to determine whether any members of its microbiome could colonize axenic larvae and rescue development. As most bacterial community members

are aerobes, we plated homogenates of conventionally reared eggs, larvae and adults on various media and then sequenced 16S rDNA products from individual colonies. This resulted in isolation of several bacterial strains in genera identified by pyrosequencing (Table A.2). We focused our recolonization assays on isolated strains of *Acinetobacter*, *Aeromonas*, *Aquitalea*, *Chryseobacterium*, *Microbacterium* and *Paenibacillus*, because we also developed PCR markers that amplified 16S rDNA products for each, but did not amplify any of the other bacterial genera we isolated (Table A.1). With the exception of *Microbacterium*, each of these strains rescued development of axenic first instars as measured by the proportion of larvae that developed into adults and the number of days required for development (Figure 2.5). PCR assays of individual mosquitoes indicated that each bacterial strain except *Microbacterium* was present in larvae after moulting to the fourth instar and also was present in newly emerged adults (Figure 2.5). Each strain detected in adults was also viable as evidenced by the ability to recover and culture from homogenates of adults.

As multiple community members rescued development, we assessed whether a non-community species had the same effect. For this experiment, we used the DH5α strain of *Escherichia coli* because previous studies showed that *E. coli* has never been identified as a gut community member in mosquitoes but can colonize under laboratory conditions (Pumpuni et al. 1996). Our results showed that cultures containing axenic

larvae, sterilized diet and *E. coli* developed into adults (Figure 2.5). PCR data also confirmed that *E. coli* colonized axenic larvae and was transstadially transmitted to adults (Figure 2.6). Thus, several strains of bacteria colonized axenic *Ae. aegypti* and rescued development.

Developmental rescue depends upon living bacteria

Heat-inactivated bacteria from the aquatic environment of conventionally reared *Ae. aegypti* did not rescue development of axenic larvae (Figure 2.5A). Inoculating cultures of axenic larvae with each strain of bacterium in the absence of sterilized standard diet also resulted in no rescue, although ~80% moulted to second instars before dying. To address whether the presence of diet and bacteria in water produced factors required for larval development, we produced bacteria-conditioned water by filter-sterilizing water from cultures of conventionally reared *Ae. aegypti* larvae and water containing individual bacterial isolates plus sterilized diet. All axenic larvae placed in bacteria-conditioned water plus new sterilized diet died as first instars (Figure 2.5A). We assessed whether dead bacteria failed to rescue development because only living bacteria stimulate larvae to feed. This was tested by providing axenic and non-sterile *Ae. aegypti* first instars heat-killed *E. coli* labelled with FITC or sterilized diet labelled with AO. We then quantified consumption of each by measuring fluorescence in the gut of

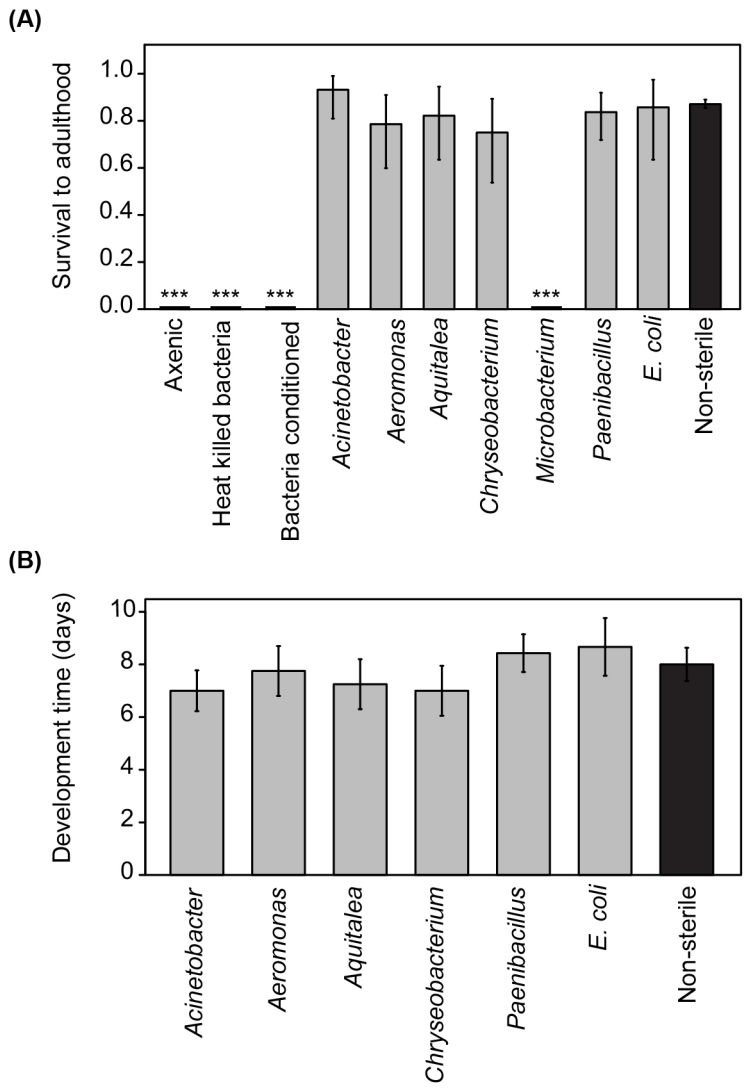


Figure 2.5. Several bacterial isolates from *Aedes aegypti* and *Escherichia coli* rescue development of axenic larvae. (A) Proportion of axenic first instars that developed into adults when fed: sterilized diet only (Axenic), heat-killed bacteria plus sterilized diet (Heat-killed bacteria), sterilized diet in bacteria-conditioned water (Bacteria conditioned) or sterilized diet plus different bacterial isolates. Non-sterile larvae fed sterilized diet served as the positive control. A minimum of 40 larvae per treatment was assayed. Columns present mean values with 95% confidence intervals for each treatment. *** indicates a significant difference for a given treatment relative to the positive control as determined by a post hoc Dunnett's test ($P < 0.0001$). (B) Development time of axenic larvae to adulthood when fed sterilized diet and different bacteria. Non-sterile larvae served as the positive control. Columns present mean values with 95% confidence intervals for each treatment. ANOVA detected no differences between treatments.

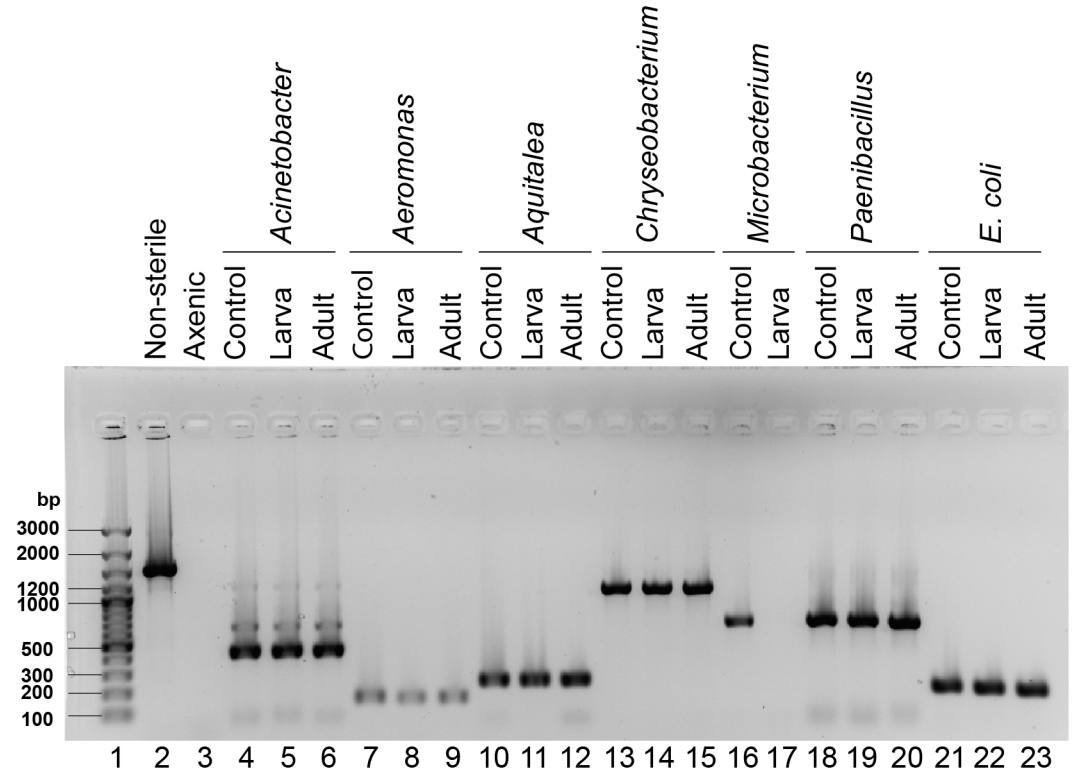


Figure 2.6. Most bacterial isolates colonized axenic *Aedes aegypti*. Axenic first instars were fed sterilized diet plus the indicated bacterial isolate. DNA was then isolated from an individual and used as template with universal or taxon-specific primers. The agarose gel shows ethidium bromide-stained PCR products. Lane 1, molecular mass marker; Lane 2, universal primers plus DNA from a non-sterile first instar; Lane 3, universal primers plus DNA from an axenic first instar; Lanes 4–6, *Acinetobacter*-specific primers plus template from *Acinetobacter* (control), a fourth instar or an adult. The same treatments are then shown for *Aeromonas* (Lanes 7–9), *Aquitalea* (Lanes 10–12), *Chryseobacterium* (Lanes 13–15), *Microbacterium* (Lanes, 16, 17), *Paenibacillus* (Lanes 18–20) and *Escherichia coli* (Lanes 21–23). At least 10 individuals were examined for each treatment with all outcomes being identical to what is presented in the figure.

individual larvae at 2-h intervals for 8 h. Our results showed that axenic and non-sterile first instars consumed dead bacteria and axenic diet at similar rates but only the latter grew, moulted and developed into adults (Figure A.3).

2.5 Discussion

Previous studies report that field-collected adult mosquitoes contain low diversity but variable bacterial communities consisting primarily of gram-negative aerobes and facultative anaerobes (Boissiere et al. 2012; Osei-Poku et al. 2012). Wang et al. (2011) reported higher diversity communities in *Anopheles gambiae* than found in other culture-independent studies, but a large proportion of the OTUs reported were represented by only one or a few reads, which suggests diversity was lower than indicated. Relative to vertebrate gut communities, low diversity aerobic communities have also been described from other holometabolous insects including *Drosophila melanogaster* (Shin et al. 2011; Wong et al. 2011), honeybees and related pollinators (Martinson et al. 2011; Engel et al. 2012), and ants (Sanders et al. 2014).

The first goal of this study was to determine whether two closely-related culicines (*Aedes aegypti*, *Georgecraigius atropalpus*) and an anopheline (*An. gambiae*) have similar bacterial communities when fed the same diet and reared in the same room. We then used this information to gain insights about transstadial transmission and the function of the microbiome. Given these objectives, we decided that pyrosequencing whole-body samples from multiple individuals was more appropriate for comparing the communities in these laboratory-reared species than dissecting guts from individual larvae and adults. However, when considered in relation to our functional data and

other culture-independent studies of microbiota in the mosquito literature (Wang et al. 2011; Boissiere et al. 2012; Osei-Poku et al. 2012), most if not all of the bacterial taxa we identified in our mosquito samples are likely members of the gut community.

As each species was reared identically in the same location, we expected the community of bacteria in our larval rearing pans would be similar. Our results fully support this and also indicate that the aquatic habitat strongly influences the bacterial community in larvae of each species. However, our results also suggest that phylogenetically distant *Ae. aegypti* and *An. gambiae* larvae contain communities more similar to one another than to *G. atropalpus*. With only three mosquito species, we recognize the functional significance of this result must be viewed cautiously. Nonetheless, they suggest that differences in the composition of the gut microbiome may play a role in autogenous reproduction. Future studies that include replicate sequencing libraries, a larger sample of autogenous and anautogenous species, and functional studies will be needed to fully assess whether the trends observed here are biologically significant.

While community diversity is low in field-collected mosquitoes (Osei-Poku et al. 2012), prior studies report that diversity is lower still in laboratory-reared *An. gambiae* (Boissiere et al. 2012). A similar pattern is also seen in *D. melanogaster* (Corby-Harris et al. 2007; Shin et al. 2011; Staubach et al. 2013). Field and laboratory populations of

An. gambiae further show differences in community composition with dominant laboratory taxa including Flavobacteriaceae being less abundant in field-collected individuals (Boissiere et al. 2012). Flavobacteria were common in our water communities and in larvae of *Ae. aegypti* and *An. gambiae*. Other genera we identified, including *Chryseobacterium*, *Elizabethkingia*, *Pseudomonas*, *Nisseria* and *Enterobacter*, are also reported to be mosquito gut community members (DeMaio et al. 1996; Dong et al. 2009; Chouaia et al. 2010; Cirimotich et al. 2011; Djadid et al. 2011; Oliveira et al. 2011; Wang et al. 2011; Osei-Poku et al. 2012; Bahia et al. 2014). Absent from our samples, however, were any Alphaproteobacteria, including the genus *Asaia*, which have been identified from anopheline and culicine mosquitoes and shown to be horizontally and vertically transmitted (Favia et al. 2007; Chouaia et al. 2010).

Although larval mosquitoes expel a large number of bacteria during moulting and metamorphosis (Moll et al. 2001), our results indicate that many community members in *Ae. aegypti* are transstadially transmitted. We did not investigate whether consumption of water from the larval habitat by newly emerged adults might alter the adult bacterial community. Nonetheless, our findings support an important role for transstadial transmission in colonizing the adult gut and support previous data that circumstantially suggest adult *Anopheles* acquire their gut community from larval breeding sites (Boissiere et al. 2012). The reduction in OTUs in NBF adults and

alterations in bacterial community composition following a bloodmeal are also consistent with patterns seen in other studies (Oliveira et al. 2011; Wang et al. 2011). As noted above, *Asaia* can be transmitted vertically through mosquito eggs as can *Wolbachia* (McMeniman et al. 2009). We detected no bacteria in mosquito eggs from our cultures after surface sterilization. However, we did detect the two dominant genera present in *Ae. aegypti* adults after blood feeding (*Chryseobacterium* and *Delftia*) on the surface of eggs, which could provide a weak mode of vertical transmission between generations.

In the second part of our study, we examined the role of gut microbiota in mosquito development. Antibiotic treatment was previously shown to slow larval development of mosquitoes, while studies with *Anopheles stephensi* indicate *Asaia* rescue antibiotic-induced delays (Chouaia et al. 2012). Our results in contrast reveal a much more profound role for the microbiome in mosquito biology. The inability of axenic larvae to develop is not due to a failure to feed or nutrient scarcity given our results showing that non-sterile larvae develop normally when fed sterilized diet. Yet, each species is rescued by bacteria from the aquatic habitat of conventionally reared larvae, while *Ae. aegypti* is rescued by several different members of the bacterial community and *Escherichia coli*. Thus, even though gut community composition can be highly variable in mosquitoes, our results strongly suggest most if not all species require gut bacteria to develop whether from the laboratory or field. This finding together with our

data showing the importance of transstadial transmission in shaping the gut community of adults also has translational implications in management of vector species.

Unknown currently is the underlying mechanism(s) for our results which show living bacteria must be present in the gut for mosquitoes to develop but also indicate several different bacterial species rescue development of gnotobiotic larvae. One option is one or more conserved bacterial products provide signalling cues that regulate growth processes in larvae. Axenic *D. melanogaster* provided a conventional diet are not developmentally arrested but do exhibit delays in growth and reductions in size that are rescued by two members of the gut community (*Acetobacter pomorum* or *Lactobacillus plantarum*) (Shin et al. 2011; Storelli et al. 2011). Rescue by *A. pomorum* is associated with the periplasmic oxidative respiratory chain initiated by pyrroloquinoline quinone-dependent alcohol dehydrogenase (PQQ-ADH), which stimulates insulin signalling in larvae by unknown means (Shin et al. 2011; Lee & Brey 2013). *L. plantarum* in contrast appears to stimulate the larval gut to assimilate protein in the diet, which activates target of rapamycin signalling (Storelli et al. 2011). Rescue by *A. pomorum* and *L. plantarum* also only occurs if living bacteria are present in the fly gut. Diet and bacteria could interact to alter oxygen tension, pH or other physical parameters, which could affect signalling activity in mosquitoes and possibly pathogen infection and transmission by adults. Gut bacteria could also potentially alter other conditions given findings that

Wolbachia alters DNA methylation and gene expression patterns in *Ae. aegypti* (Ye et al. 2013; Zhang et al. 2013).

2.6 Acknowledgements

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CHAPTER 3

MOSQUITOES HOST COMMUNITIES OF BACTERIA THAT ARE ESSENTIAL FOR DEVELOPMENT BUT VARY GREATLY BETWEEN LOCAL HABITATS[2]

[2]Coon, K.L., Brown, M.R. and M.R. Strand. 2016. *Molecular Ecology* 25:5806-5826. Reprinted here with permission of the publisher.

3.1 Abstract

Mosquitoes are insects of interest because several species vector disease-causing pathogens to humans and other vertebrates. We previously reported that mosquitoes from long-term laboratory cultures require living bacteria in their gut to develop, but development does not depend on particular species of bacteria. Here, we focused on three distinct but interrelated areas of study to better understand the role of bacteria in mosquito development by studying field and laboratory populations of *Aedes aegypti*, *Aedes albopictus* and *Culex quinquefasciatus* from the southeastern United States. Sequence analysis of bacterial 16S rRNA gene amplicons showed that bacterial community composition differed substantially in larvae from different collection sites, whereas larvae from the same site shared similarities. Although previously unknown to be infected by *Wolbachia*, results also indicated that *Ae. aegypti* from one field site hosted a dual infection. Regardless of collection site or factors like *Wolbachia* infection, however, each mosquito species required living bacteria in their digestive tract to develop. Results also identified several concerns in using antibiotics to eliminate the bacterial community in larvae in order to study its developmental consequences. Altogether, our results indicate that several mosquito species require living bacteria for development. We also hypothesize these species do not rely on particular bacteria

because larvae do not reliably encounter the same bacteria in the aquatic habitats they develop in.

Keywords: development, insect, microbiota, *Wolbachia*

3.2 Introduction

Most multicellular animals host communities of microbes in their digestive tract. One important area of study is to understand how animals acquire these microbes and the factors that influence their composition. This is because several studies show that the gut microbiota and its composition have wide-ranging effects on the physiology of different vertebrates and invertebrates (Buchon et al. 2013; Lee & Brey 2013; Sommer & Backhed 2013; Blaser 2016; Goodrich et al. 2016).

Among insects, some groups including termites (Blattodea), bees and ants (Hymenoptera), and select Heteroptera have specialized gut microbiota that are maintained by direct transfer between individuals (Fukatsu & Hosokawa 2002; Ohkuma & Brune 2011; Anderson et al. 2012; Martinson et al. 2012). Others including certain moths (Lepidoptera) and fruit flies (Diptera: Drosophilidae) acquire most if not all of their gut microbiota each generation from the environment (Robinson et al. 2010; Chandler et al. 2011; Tang et al. 2012). In the case of mosquitoes (Diptera: Culicidae), all species are aquatic as larvae, feeding on detritus, microorganisms and small

invertebrates, while adults are terrestrial with both sexes usually feeding on nectar (Clements 1992; Merritt et al. 1992). Adult females of most species also must feed on vertebrate blood to produce eggs, which underlies why many mosquitoes are important disease vectors (Clements 1992). Deep sequencing of bacterial 16S rRNA gene amplicons from select species shows that larvae contain a subset of the operational taxonomic units (OTUs) in their aquatic environment, while some but not all of the OTUs in larvae are also in adults (Wang et al. 2011; Boissiere et al. 2012; Coon et al. 2014; Gimonneau et al. 2014). Controlled experiments further indicate that mosquito larvae contain no bacteria if they hatch from surface-sterilized eggs and are maintained in a sterile environment, while also showing that most OTUs in adults overlap with those in larvae (Coon et al. 2014). Altogether, these data support that mosquito larvae acquire their gut microbiota from the water in which they feed and that some community members in larvae persist in adults.

Variability in the microbiota of mosquitoes has been detected within and between species from different geographic locations (Boissiere et al. 2012; Osei-Poku et al. 2012; Gimonneau et al. 2014; Duguma et al. 2015; Buck et al. 2016; Muturi et al. 2016). In contrast, less is known about community composition on finer geographic scales, which is relevant to many vector species that develop as larvae in small volume containers, which are often located in close proximity to one another in urban habitats. The issue of

variability in bacterial community composition is also functionally important from the perspective of mosquito development. Most mosquitoes moult through four instars before metamorphosis into adults when fed a nutritionally complete diet under conventional (non-sterile) rearing conditions (Clements 1992). However, our own recent studies of three species (*Aedes aegypti*, *Aedes* (=*Georgecraigius*) *atropalpus* and *Anopheles gambiae*) from long-term laboratory cultures show that axenic larvae with no bacteria die as first instars when fed a nutritionally complete diet (Coon et al. 2014). Axenic larvae of each species also die as first instars if fed dead bacteria plus diet or diet preconditioned by living bacteria before feeding. Recolonization with a mixed community of bacteria from conventional cultures, however, rescues development. Experiments with *Ae. aegypti* further show that several members of the laboratory bacterial community including an *Acinetobacter*, *Aeromonas*, *Aquitalea* and *Chryseobacterium* or the non-community member *Escherichia coli* individually colonize the gut, which produces monoassociated, gnotobiotic larvae that develop normally (Coon et al. 2014). Resulting adults also show no morphological defects or reductions in fitness as measured by development time, size and fecundity (Coon et al. 2014, 2016a). Thus, each of the species studied by Coon et al. (2014) required living bacteria for development, yet development did not depend on a particular species or community assemblage because several different bacteria rescued development of gnotobiotic larvae.

In contrast, a study that used antibiotics to eliminate the gut microbiota from *Anopheles* mosquitoes reported delays in growth but larvae nonetheless survived and developed into adults (Chouaia et al. 2012). Older studies also report variable effects on mosquito development after perturbing the microbial communities in larval habitats (Hinman 1930; Rozeboom 1935; Ferguson & Micks 1961; Jones & DeLong 1961; Chao et al. 1963). Thus, unlike our own results, some studies in the literature suggest living bacteria in the gut are not required for mosquito larvae to develop. One explanation for these differing conclusions is that mosquitoes vary in their requirement for bacteria as a function of species or rearing conditions that could also affect the communities of bacteria that are naturally present. Another factor of potential importance is that vertically transmitted intracellular bacteria like *Wolbachia* infect some mosquitoes and can affect fitness both positively and negatively (Brownlie et al. 2009; Ross et al. 2016). However, as Coon et al. (2014) examined laboratory populations of mosquitoes that were not infected by *Wolbachia* or other intracellular bacterial symbionts, this study could not assess whether *Wolbachia* potentially affect the importance of gut bacteria for development. The third possibility is that surface sterilization of eggs and antibiotic treatment do not equivalently eliminate bacteria from mosquitoes, which underlies why these two approaches have led to differing conclusions about the functional role of the gut microbiota in larvae.

The overall goal of this study, therefore, was to determine whether any of the factors discussed above affect whether mosquito larvae require living bacteria in their digestive tract for development. To meet this goal, we studied field and laboratory populations of *Aedes aegypti*, *Aedes albopictus* and *Culex quinquefasciatus* from the southeastern United States (US). Each of these species has a worldwide distribution in subtropical and tropical regions (Rey et al. 2006; Leonel et al. 2015). *Aedes aegypti* and *C. quinquefasciatus* were introduced into North America from Africa centuries ago, while *Ae. albopictus* was introduced from Asia in the 1980s (Huang et al. 2011; Bargielowski & Lounibos 2014). *Aedes albopictus* has also altered the range of *Ae. aegypti*, which along the east coast of the United States is infrequently collected outside of Florida (Bargielowski & Lounibos 2014; Hahn et al. 2016). In the first part of our study, we used 16S rRNA gene analysis to characterize bacterial diversity in larvae from field versus laboratory cultures. We also examined larvae from different field sites to assess the variability of bacterial community composition between breeding sites that were in close proximity to one another. Second, we assessed whether larvae from each species differed in their requirement for living bacteria as a function of being from the laboratory or field, the composition of the bacterial community where larvae were collected from, or *Wolbachia* infection. Third, we compared the efficacy of surface-sterilizing eggs and antibiotic treatment in eliminating bacteria from larvae and their

effects on development. Results showed that bacterial diversity in larvae differed substantially between collection sites. However, each species required living bacteria in their digestive tract for development regardless of where they came from or whether they were infected with *Wolbachia*. Antibiotic treatment in contrast did not fully eliminate bacteria, which results in larvae that develop. High concentrations of some antibiotics also adversely affected larvae independent of their impact on bacteria.

3.3 Materials and Methods

Field collections and laboratory cultures

We assessed bacterial diversity in *Aedes aegypti*, *Aedes albopictus* and *Culex quinquefasciatus* by collecting samples from six field sites and laboratory cultures. One field site was located in Athens, GA, and contained *Ae. albopictus* and *C. quinquefasciatus* (Figure 3.1). Five others were located in Jacksonville, FL, USA, with one containing both *Ae. aegypti* and *C. quinquefasciatus*, three containing only *Ae. aegypti* and one containing only *C. quinquefasciatus* (Figure 3.1). Each site was a small container habitat that was sampled 2 or 3 times over the breeding season by collecting: (i) 50 ml of water in a sterile Falcon tube (BD Diagnostics) that was transferred to ice and (ii) mosquito larvae in 300 ml of water that were placed in a 500-ml container.

Samples were taken to the laboratory where larvae were counted in a laminar hood and identified to species using defined morphological characters (Breeland & Loyless 1989). Water samples were centrifuged at 5000 x *g* for 30 min at 4 °C, and the resulting pellet was stored at −20 °C until DNA isolation. Almost all larvae (>98%) collected from the field sites we sampled were *Ae. aegypti*, *Ae. albopictus* or *C. quinquefasciatus*. As previously noted, results from prior studies showed that laboratory cultures of three species (*Ae. aegypti*, *Ae. atropalpus* and *Anopheles gambiae*) contained no *Wolbachia*, and eggs that hatched from surface-sterilized eggs contained no bacteria as measured using culture and PCR-based assays (Coon et al. 2014). This latter result indicated that all of the OTUs identified by deep sequencing in laboratory-reared *Ae. aegypti* were acquired from the environment through larval feeding, while also suggesting most if not all of the OTUs in conventionally reared larvae were gut community members (Coon et al. 2014). Prior studies also showed that the small size of mosquito larvae and corresponding small number of bacteria that are present ($\sim 10^{3-4}$) (Coon et al. 2016a) often do not yield sufficient DNA template for library construction. Thus, for this study, pooled samples were prepared by collecting 15 larvae (fourth instars) per species, site and collection date. Each pool was then rinsed in 70% EtOH, dried and stored at −20 °C until use for DNA isolation. The remaining larvae were kept in their habitat water until pupation. Pupae were then surface-sterilized and allowed to emerge in sterile cages

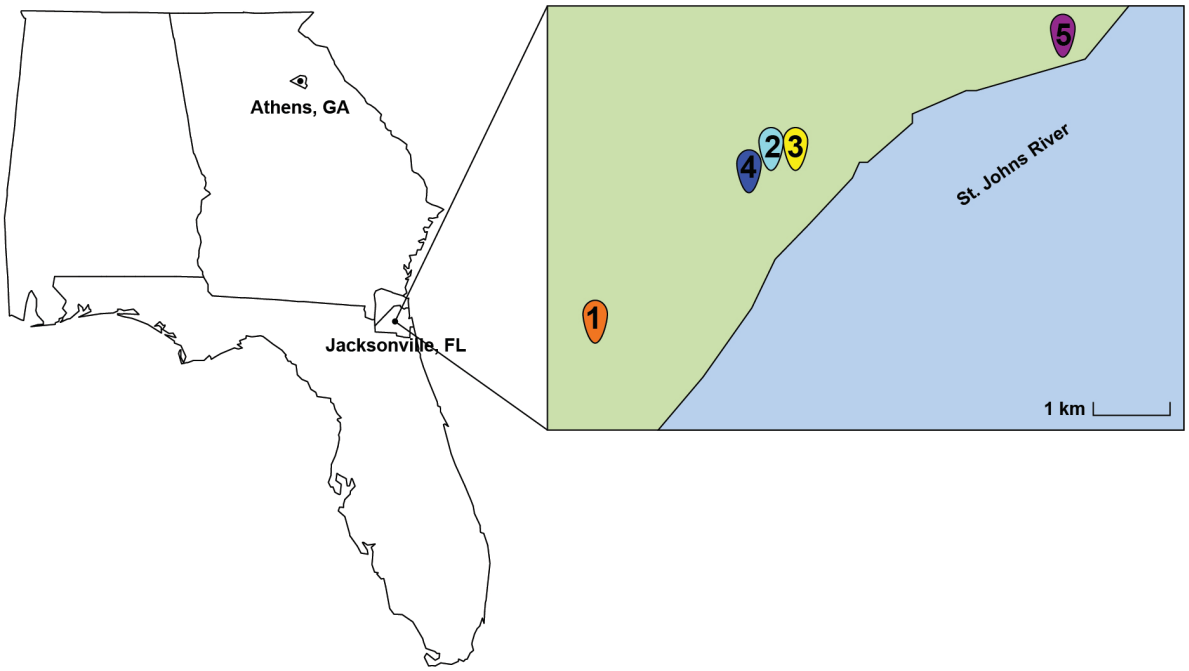
(Coon et al. 2014). Three adult female *Ae. aegypti* from site 1 in Jacksonville, FL were rinsed in 70% EtOH, dried and stored at −20 °C for DNA isolation. Ribosomal internal transcribed spacer region 1 (ITS1) from these adults was PCR-amplified using ITS1A and ITS1B primers (Beebe et al. 2000) followed by comparison with the known ITS1 sequence for *Ae. aegypti* (Wesson et al. 1992). Other adults were maintained at 27 °C and a 16-h light:8-h dark photoperiod and fed 10% sterile sucrose in water. Day 3 females were blood fed on a surface-sterilized rat and then allowed to oviposit onto moist filter paper. Eggs were collected within 24 h of being laid and either stored in humidified containers (*Ae. aegypti*, *Ae. albopictus*) or immediately surface-sterilized and hatched (*C. quinquefasciatus*) for use in developmental assays.

Laboratory cultures for each species were maintained in an insectary that was 2 km from the Athens, GA, field site. The *Ae. aegypti* culture was established in 1974 from offspring collected in Athens, GA (UGAL strain) (Foster & Lea 1975). The *Ae. albopictus* and *C. quinquefasciatus* cultures were obtained in 2012 from the Centers for Disease Control (Atlanta, GA, USA) from offspring collected in Johannesburg, South Africa, and Keyport, NJ, USA, respectively (Marcombe et al. 2014; Cornel et al. 2003). Larvae were maintained in covered rearing pans containing distilled water and fed a nutritionally complete, standard diet of ground rat chow (Purina): lactalbumin (Sigma): torula' yeast (MP Biomedicals) (1:1:1). Pupae were transferred to cages for adult

emergence, while females were blood fed as described above to produce eggs. Water and larvae from the laboratory were collected for DNA isolation as described above.

Bacterial 16S rRNA library construction and sequencing

DNA was isolated from samples using the Gentra Puregene Yeast/Bacteria Kit (Qiagen). All samples were processed at the same time to eliminate potential batch contamination effects. As our primary goal was to assess bacterial diversity within and between sites, bacterial 16S rRNA gene V3-V4 variable regions were PCR-amplified using the universal primers 341F and 785R (Faircloth & Glenn 2012; Klindworth et al. 2013) containing multiplex identifier sequences (Table B.1). PCR amplifications were performed in triplicate 20 μl reactions containing 10 ng of template DNA, 0.4 U of iProof High-Fidelity polymerase (Bio-Rad), 1× polymerization buffer, 200 μm dNTPs and 0.2 μm of each primer. Reactions using template from blank DNA extractions served as a negative control. Reaction conditions were as follows: initial denaturation cycle of 98 °C for 30 s, followed by 25 cycles at 98 °C for 10 s, 55 °C for 15 s and 72 °C for 15 s, and a final extension step at 72 °C for 5 min. Products were visualized on 1.2% agarose gels, pooled, purified with the MinElute PCR purification kit (Qiagen) and eluted in 11 μl of EB buffer. Pooled amplicons served as template for a second round of PCR to attach Nextera indices and adapters (Faircloth & Glenn 2012). Products were



Site	Sampling date	Sample source	Mosquito species	Total sequences	Number of OTUs	Species richness	Chao1	Shannon index
Jacksonville, FL								
Bird bath (Site 1)	6/23/14	Water		136,941	346	315	354.667	5.921
30.294377, -81.709633		Larvae	*Ae. aegypti* (30)	143,565	517	416	476.339	4.964
Dirty w/ decomposing			*C. quinquefasciatus* (70)	123,277	523	415	496.148	5.452
plant matter,leaf litter	7/21/14	Water		110,190	568	492	569.609	5.988
		Larvae	*Ae. aegypti* (70)	139,119	509	411	469.400	5.421
			C. quinquefasciatus (30)	131,173	440	342	419.025	4.926
	9/15/14	Water		151,366	457	341	395.886	5.311
		Larvae	*Ae. aegypti* (50)	105,299	370	299	409.676	3.975
Recycling bin (Site 2)	6/23/14	Water		147,937	330	226	302.250	4.180
30.30302, -81.698464		Larvae	*Ae. aegypti* (20)	186,279	258	149	239.588	2.262
Clear, recyclable waste	9/15/14	Water		147,021	448	368	418.324	6.082
(glass/cardboard)		Larvae	*Ae. aegypti* (20)	159,666	429	291	397.344	4.034
Flower pot (Site 3)	7/21/14	Water		144,246	211	188	243.111	4.937
30.303127, -81.698665		Larvae	*Ae. aegypti* (20)	117,921	428	343	432.071	4.784
Empty planter, clear w/	9/15/14	Water		119,362	452	394	451.000	5.991
residual sediment		Larvae	*Ae. aegypti* (20)	108,519	556	471	582.300	5.001
Flower pot (Site 4)	7/21/14	Water		77,254	510	466	522.692	6.504
30.303143, -81.699313		Larvae	*Ae. aegypti* (20)	45,354	370	356	407.333	6.066
Plant saucer w/ overflow	9/15/14	Water		95,523	628	554	644.000	7.003
(basil plant)		Larvae	*Ae. aegypti* (20)	119,014	535	471	533.255	5.423
Memorial Park Fountain (Site 5)	7/21/14	Water		148,134	248	207	234.188	5.591
30.310097, -81.679246		Larvae	*C. quinquefasciatus* (100)	38,735	85	85	96.000	4.278
Drained; eutrophic w/	9/15/14	Water		158,193	295	215	325.200	4.825
human waste,oil, water mold		Larvae	*C. quinquefasciatus* (100)	176,289	335	229	368.333	4.639
Athens, GA								
Residential Fountain	7/7/14	Water		187,534	380	235	319.091	3.863
33.944131, -83.38726		Larvae	*Ae. albopictus* (50)	166,002	457	345	410.395	4.147
Broken, still water; murky w/			*C. quinquefasciatus* (50)	148,658	377	273	393.750	4.871
sediment, leaf litter, algal bloom	9/23/14	Water		166,649	472	327	438.250	5.456
		Larvae	*Ae. albopictus* (50)	127,349	473	368	507.794	4.488
			C. quinquefasciatus (50)	185,457	394	293	344.692	4.971
Laboratory								
33.947941, -83.372955	9/30/14	Water		170,376	214	180	189.000	4.479
		Larvae	*Ae. aegypti*	114,344	205	172	198.300	4.625
		Water		168,216	224	173	190.500	4.462
		Larvae	*Ae. albopictus*	138,838	253	213	195.882	4.107
		Water		191,711	218	162	228.000	5.171
		Larvae	*C. quinquefasciatus*	146,397	133	105	113.750	3.145

Figure 3.1. Collection sites and sequencing statistics for the 16S rRNA libraries prepared from water and mosquito larvae. The upper portion of the figure shows the locations of the collection sites in Georgia and Florida. The lower portion of the figure summarizes the sites, sampling dates and mosquito species that were collected. For each collection site, latitude and longitude coordinates are indicated along with a general description of the habitats, which were all containers of variable size. Numbers in parentheses in the column named 'Mosquito species' indicate the approximate number of larvae that were present per 300 ml of water. This provides a sense of larval density between sites and collection dates. Number of reads for each library after quality filtering, number of OTUs, and indices for species richness and diversity (Chao 1, Shannon's H) are also presented.

again visualized on agarose gels and repurified before quantification using a Qubit dsDNA HS assay and fluorometer (Invitrogen). Three libraries were constructed and sequenced per sample (i.e. three technical replicates) using different barcode sequence combinations to prevent PCR bias. For each replicate PCR, negative controls produced no amplicon indicating no contamination of reagents. Altogether, six field sites sampled multiple times and three laboratory cultures resulted in 36 samples. Three technical replicates per sample thus resulted in 108 sequencing libraries. Barcoded products were combined in equimolar amounts with libraries repurified using a SPRI plate and Sera-Mag Speed beads prior to paired-end sequencing (2 × 300 bp) on half of an Illumina MiSeq lane by the University of Georgia Genomics Facility. Triplicate libraries from three adult *Ae. aegypti* females that emerged from larvae collected at site 1 in Jacksonville, FL, on 21 July 2014 were also paired-end sequenced.

Amplicon data analyses

Sequenced reads were trimmed at any site of two sequential bases receiving a quality score <Q30, and paired-end reads were merged using PEAR (Zhang et al. 2014). Read filtering criteria included the following: no ambiguous base calls or barcode/primer errors, all bases must have a PHRED equivalent score of 30 or higher (per base error rate of 0.1%), and reads must be between 440 and 484 bp in length. Quality-filtered reads were demultiplexed using an in-house Perl script and assigned to operational taxonomic units (OTUs) at a 97% identity threshold using the quantitative insights into microbial ecology (QIIME) version 1.7.0 (Caporaso et al. 2010b) USEARCH 6.1 wrapper (Edgar 2010). Chimeric sequences were identified and removed using UCHIME (Edgar et al. 2011). Taxonomic classification was assigned against the latest Greengenes database (http://greengenes.lbl.gov/cgi-bin/nph-index.cgi) using the QIIME-based RDP Bayesian classifier with a 0.80 confidence threshold. OTUs making up <0.005% of sequence libraries were filtered out of our analysis (Bokulich et al. 2013). Sequence alignment was performed by PyNAST (Caporaso et al. 2010a), and phylogenetic tree construction was performed using FastTree (Price et al. 2009).

For each sample, we first compared calculated indices for species richness and diversity (Chao1, Shannon's H) within each technical replicate library to assess whether between-replicate variation could affect alpha diversity estimates. One-way ANOVA

detected no significant differences between replicated libraries for each measure ($P > 0.05$) (Table B.2). We also compared beta diversity using the unweighted UniFrac distance and Bray–Curtis index of dissimilarity between pairs of samples within a given replicate. One-way ANOVA again detected no differences between technical replicates for any sample or either metric ($P > 0.05$) (Table B.2). We therefore combined the three technical replicate libraries for each sample for diversity analyses, which were conducted on data rarefied to 38,735 sequences per sample. Separate analyses of variance (ANOVAs) were performed using R (http://www.r-project.org/) to compare alpha diversity (species richness, Chao1 and Shannon's H diversity indices) between sites and mosquito species.

We performed a principal coordinates analysis (PCoA) on Bray–Curtis distances using QIIME (Lozupone et al. 2007). A multivariate analysis of variance (MANOVA) was run on the first three PCoA vectors across all larval libraries to determine whether site, mosquito species or collection date were significantly associated. We then performed univariate ANOVAs on significant MANOVA factors to examine which axis or axes drove the differences visible in the PCoA plots. Separate MANOVAs followed by univariate ANOVAs were also run using only the larval libraries for the sites in Jacksonville, FL, or the two sites that contained more than one mosquito species. We assessed whether unweighted UniFrac distances were smaller for larval libraries of

species within versus between sites using Monte Carlo t-tests (9,999 simulations). The same approach was used to assess whether UniFrac distances were smaller between water and larval libraries from a given site at late versus early sampling dates. We tested for differences in taxon abundance across sample groups using the QIIME script otu_category_significance.py. All MANOVAs and ANOVAs were run in R (http://www.r-project.org/) and analysed with Pillai's Trace as the test statistic (Sullam et al. 2012).

We used oligotyping of 16S ribosomal RNA gene amplicons to further investigate bacterial diversity within the six dominant phyla detected by OTU clustering. This was performed separately at the phylum level for five taxa (Actinobacteria, Bacteroidetes, Firmicutes, Cyanobacteria and Verrucomicrobia), while for Proteobacteria, each class (Alphaproteobacteria, Betaproteobacteria, Gammaproteobacteria and Deltaproteobacteria) was analysed separately. Taxonomic assignments were made using quality-filtered reads against the latest Greengenes database using the RDP classifier with a 0.80 confidence threshold. Sequences corresponding to each phylum or class were extracted using an in-house python script and the o-pad-with-gaps script was used to eliminate any length variation between reads.

Oligotyping, which uses Shannon (1948) entropy to detect the amount of diversity at each nucleotide position, was conducted as outlined by Eren et al. (2013).

Entropy values for nucleotide positions ranged from 0 to 2 with values ≥1.0 indicating greater bacterial diversity. Oligotypes were generated using the first position with the highest entropy value followed by further decomposition using user-defined nucleotide selection. Between 25 and 85 positions were chosen for each of the nine data sets until no peaks were left unresolved. Peaks ≤0.2 were considered background noise. The value for parameter 's' (the minimum number of samples in which an oligotype is present) was set to 2 to correct for technical errors due to sequencing, parameter 'A' was set to 0.0005% (the minimum total abundance of an oligotype in all samples) of the total reads for each data set to match the parameter set for OTU clustering, and parameter 'M' (the minimum count of the most abundant unique sequence in an oligotype) was set to 137 which corresponded to the average number of reads per sample divided by 1000 (Eren et al. 2013). Oligotype representative sequences (i.e. the most abundant sequence within an oligotype) were classified using the RDP classifier with a 0.8 confidence threshold. For determining percent alignment scores, the sequences corresponding to individual oligotypes were extracted from the OLIGO-REPRESENTATIVES directory and aligned using MAFFT (Katoh et al. 2005).

Multilocus sequence typing of *Wolbachia* from *Aedes aegypti*

Wolbachia from *Ae. aegypti* was genotyped by multilocus sequence typing (MLST) of five housekeeping genes (*gatB*, *coxA*, *hcpA*, *fbpA* and *ftsZ*) (Baldo et al. 2006). Each gene was amplified using primer sets and reaction conditions for double AB-infected individuals as outlined by the *Wolbachia* MLST website (http://pubmlst.org/wolbachia/info/amp_seq_double.shtml). PCR products were visualized on 1% agarose gels using ethidium bromide followed by purification (PCR Purification Kit, Qiagen) and bidirectional sequencing (Eurofins MWG Operon). Sequences were concatenated, aligned and trimmed followed by analysis against the *Wolbachia* MLST database (http://pubmlst.org/wolbachia/). PHYML version 3.0 with the SPR search option was used for phylogeny construction, while branch support was estimated by bootstrapping the data set 1,000 times (Guindon et al. 2010).

Development assays

First instars with no gut bacteria were produced from surface-sterilized eggs as previously described (Coon et al. 2014; Appendix Suppl. Methods B.1). The status of larvae was confirmed using culture and PCR assays (Appendix Suppl. Methods B.1). Standard mosquito larval rearing diet was sterilized by cobalt 60 gamma irradiation with sterility of diet confirmed by culture-based assays (Appendix Suppl. Methods B.1).

Water for sterile rearing cultures was autoclaved. Ten first instars were placed into 25-cm^2 cell culture flasks (Corning) containing 20 ml of sterile water and sterilized standard diet (2 mg). Some flasks were maintained this way while to others we added 100 µl of water from laboratory trays containing conventionally reared larvae, 100 µl of water from site 1 in Jacksonville, FL, or 2 µl of an overnight *Escherichia coli* (K-12 MG1655 strain) culture. Note that inoculating cultures with water from conventional laboratory cultures or field site 1 provided the community of bacteria that was present. Each of the above treatments was replicated a minimum of three times with the proportion of first instars that developed into adults and total development time (days) determined by inspecting cultures daily. Survival data were analysed by Fisher's exact tests followed by post hoc Bonferroni-corrected pairwise tests to compare treatments. Development times were tested for normality and equality of variances before analysis by one-way ANOVA and post hoc Tukey–Kramer honest significant difference (HSD) tests using R.

Antibiotic assays

We previously isolated, identified and cultured several members of the bacterial community in UGAL *Ae. aegypti* (Coon et al. 2014). Several members of the community in *Ae. albopictus* fourth instars collected at the Athens, GA, field site were isolated in this study by the same methods (Table B.3). Five community members from UGAL *Ae.*

aegypti (*Aquitalea*, *Aeromonas*, *Chryseobacterium*, *Comamonas* and *Sphingobacterium* spp.) and two from field-collected *Ae. albopictus* (*Bacillus* and *Aeromonas* spp.) were then used to assess their antibiotic susceptibility by standardized dilution (Wiegand et al. 2008). In brief, single colonies from overnight culture plates were suspended to 5×10^5 colony-forming units (CFUs) per ml in Luria Broth (LB) containing serially diluted ampicillin (Sigma), kanamycin (Roche), streptomycin (Sigma), chloramphenicol (Sigma), penicillin (Sigma), tetracycline (Sigma) or gentamicin (Gibco) (200 μg–2 ng/ml). Suspensions were incubated at 28 °C for 18 h. The minimal inhibitory concentration (MIC) was recorded as the lowest amount of antibiotic where no visual growth of bacteria was detected. We then placed newly hatched first instars (N = 12) from the conventionally reared UGAL *Ae. aegypti* culture into 1 ml of distilled water in a culture plate containing non-sterilized standard diet (300 μg) and the same dilution series of antibiotics. A total of 24–72 larvae per antibiotic dose were bioassayed. The number of larvae that died or moulted to the second instar after 2 or 5 days was then recorded with 2-day moulting data further analysed by Fisher's exact tests. In other replicates, we selected six larvae at 2 days post-hatching in cultures that contained either 200 μg/ml of ampicillin, kanamycin, streptomycin, chloramphenicol or penicillin, or 20 μg/ml of tetracycline or gentamicin. Larvae cultured for the same period with no antibiotic served as a positive control, while axenic larvae served as a negative control.

Larvae were then individually homogenized in Luria Broth (LB) followed by serial dilution on LB plates which were incubated at 27 °C for 72 h. The number of living bacteria per individual that could be cultured was then determined by colony count assay (Coon et al. 2016a). Lastly, axenic first instars were produced and cultured in different concentrations of the same antibiotics followed by determination of the proportion of larvae that died after 5 days. Axenic larvae cultured with no antibiotics served as the positive control with mortality data across all treatments analysed by Fisher's exact tests.

3.4 Results

Clustering analyses of amplicon data identify variable bacterial communities

Multiplex sequencing of 16S rRNA gene amplicons for all field and laboratory samples generated a total of 8.3 million reads of which 5.8 million were retained after quality filtering. Reads per sample ranged from 38,735 to 191,711 and grouped by percentage similarity into 1,253 operational taxonomic units (OTUs) at a cut-off threshold of 97% (Figure 3.1, Table B.4). However, 68 OTUs accounted for more than 50% of the total quality-filtered reads. Rarefaction curves saturated or near saturated at 3,000 sequences,

which indicated that most bacteria in each sample were captured (Figure B.1). Sample complexity varied with OTUs ranging from 211 to 628 for water and 85 to 556 for larvae. Bacterial species diversity differed by site as measured by richness ($F_{(6,29)} = 10.63$, $P < 0.0001$), Chao1 ($F_{(6,29)} = 9.672$, $P < 0.0001$) and Shannon's H indices ($F_{(6,29)} = 3.68$, $P < 0.01$) (Figure 3.1). The highest richness values were from sites in Jacksonville, FL, while the lowest were from laboratory cultures (Figure 3.1). Richness was higher in water than larvae at most sites, while alpha diversity in water and larvae at each site significantly correlated ($r = 0.72$, $P = 0.0004$).

Twenty-nine bacterial phyla were identified across all samples, but six accounted for 81% of the OTU groups at the 97% cut-off threshold: Proteobacteria (49%), Actinobacteria (11%), Bacteroidetes (12%), Firmicutes (5%), Cyanobacteria (2%) and Verrucomicrobia (2%). Classification into orders showed that larvae contained the same taxa present in the water they were collected from but relative abundance differed. To visualize these trends, we merged sample times for each collection site to produce the bar graphs shown in Figure 3.2. This showed that laboratory-reared *Aedes aegypti*, *Aedes albopictus* and *Culex quinquefasciatus* contained a greater proportion of Actinomycetales than field-collected larvae ($P = 0.0003$, after FDR correction for multiple comparisons), while *Ae. albopictus* and *C. quinquefasciatus* from the Athens, GA field site contained a higher proportion of Burkholderiales than larvae from other

sites ($P = 0.005$) (Figure 3.2). Considerable heterogeneity at the ordinal level was also observed between the field sites in Jacksonville, FL, which was striking given the proximity of most sites to one another (Figure 3.1).

PCoA using the Bray–Curtis dissimilarity index showed clustering by collection site, mosquito species and sampling date (Figure 3.3). A MANOVA across all larval libraries indicated that differences along the PCoA axes were significant for site but not species or collection date (Table 3.1A). Separate MANOVAs for only the larval libraries from Jacksonville, FL (Table 3.1B) or the three sites that contained more than one mosquito species (site 1 in Jacksonville, FL, the field site in Athens, GA, and the laboratory) (Table 3.1C) likewise indicated the bacterial communities in larvae differed by site but not collection date or species. We next calculated the unweighted UniFrac distances between samples. Pairwise analysis of all larval sequencing libraries using Monte Carlo resampling indicated that UniFrac distances differed between the same species from different sites ($P \leq 0.002$), whereas no significant differences were detected between mosquito species from the same site ($P > 0.05$) (Figure 3.4). Comparing UniFrac distances between the larval and aquatic samples from each field site also showed that distances were significantly larger at the early versus late sampling date ($P \leq 0.03$). Overall, these results indicated that the bacterial communities in water and mosquito larvae strongly differed between sites, while also suggesting that the larval and

water communities within each site were more dissimilar to one another at the early collection dates than at the late collection dates.

A final feature of note was detection of low abundance reads corresponding to *Wolbachia* in some but not all larval samples. Two *Wolbachia* OTUs were detected in all field and laboratory samples for *Ae. albopictus*, while one *Wolbachia* OTU was detected in all field and laboratory samples for *C. quinquefasciatus*. No *Wolbachia* were detected in the *Ae. aegypti* laboratory culture (UGAL), which we previously reported (Coon et al. 2014), or in *Ae. aegypti* larvae from sites 2–4 in Jacksonville, FL. However, two *Wolbachia* OTUs were detected in *Ae. aegypti* larvae from site 1 in Jacksonville, FL. Mean prevalence of *Wolbachia* across larval samples for all species was 0.35%. Detection of *Wolbachia* in *Ae. albopictus* and *C. quinquefasciatus* was expected because both are known to harbour natural infections in North America (Kittayapong et al. 2002; Zouache et al. 2009; Almeida et al. 2011; Atyame et al. 2011; Minard et al. 2014). This was not expected though for *Ae. aegypti* because no natural infection of this species by *Wolbachia* has been reported anywhere in the world (Ross et al. 2016). We therefore reared and analysed *Ae. aegypti* adults from larvae collected at Jacksonville site 1 on 21 July 2014 to assess whether they too were *Wolbachia* infected. Sequencing ITS1 domains from three adult females confirmed each was correctly identified to species. Sequencing

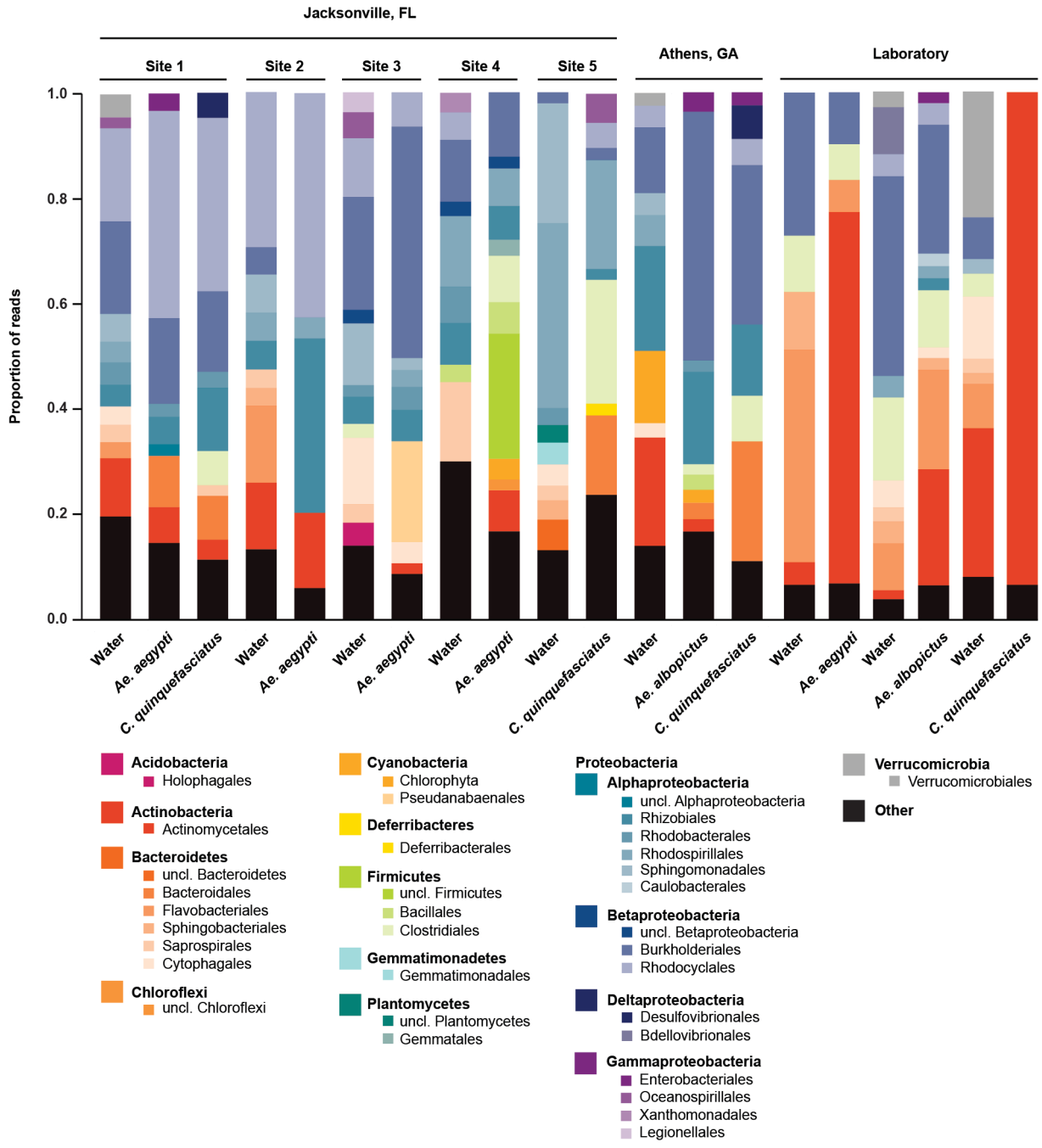


Figure 3.2. Bacteria at the levels of phylum and order in water and mosquito larvae from each collection site. Water and larval (fourth instar) libraries for each collection date were pooled for the bar graphs presented. Each bar presents the proportion of sequencing reads assigned to a given bacterial order. Only categories >2% are presented.

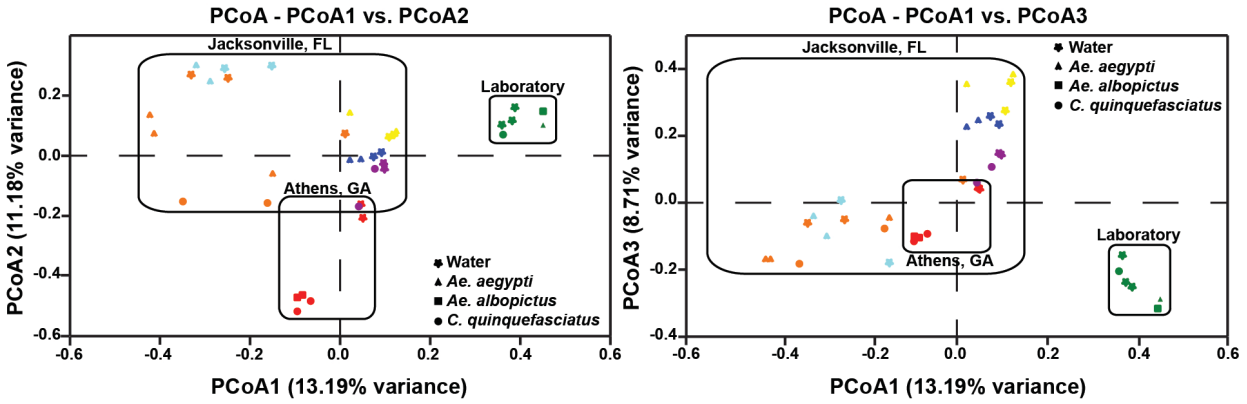


Figure 3.3. Principal coordinates analysis based on pairwise Bray–Curtis distances. Symbols are coloured by collection site (laboratory: green, Athens, GA: red, Jacksonville, FL: orange (site 1), dark blue (site 2), light blue (site 3), yellow (site 4), purple (site 5). The legends in the upper right of each plot designate sample type (water or species of larva) by symbol shape. Water and larval samples for each collection date are presented but are not given unique identifiers because date was not statistically significant (see Table 3.1).

of 16S rRNA gene amplicons from triplicate libraries produced from the same females also showed that the abundance of *Wolbachia* reads was much higher than in larvae but grouped into the same two OTUs (Figure B.2). MLST genotyping indicated one of these strains belonged to supergroup A and was related to a strain in *Ae. albopictus*, while the second was a supergroup B that clustered with strains present in a number of other insects, including *Ae. albopictus* that were collected in Thailand (Figure B.3). Non-*Wolbachia* reads in these adults grouped into 27–43 OTUs that were either identical or belonged to the same genera detected in larvae but whose relative abundance differed (Figure B.2). For example, Rhodocyclales that were abundant in larvae were reduced,

while Rhodospirillales and Burkholderiales (adults 1 and 2) or Burkholderiales and Pseudomonadales (adult 3) were elevated (Figure B.2).

Table 3.1. Effects of collection site, species and collection date on larval bacterial communities in mosquitoes.

(A) Multivariate analysis of all larval libraries					
Source of variation	Pillai's trace	Hypothesis d.f.	Error d.f.	*F*	*P*
Site	2.882	18	24	32.630	<0.0001*
Species	0.705	6	14	1.269	0.332
Sampling date	0.560	9	24	0.612	0.775
Univariate analysis of site (all larval libraries)					
	Type III SS	d.f.		*F*	*P*
Site					
PCoA1	1.160	6		29.905	<0.0001*
PCoA2	1.097	6		28.223	<0.0001*
PCoA3	0.729	6		60.672	<0.0001*
(B) Multivariate analysis of all larval libraries from only Jacksonville, FL					
Source of variation	Pillai's trace	Hypothesis d.f.	Error d.f.	*F*	*P*
Site	2.624	12	15	8.718	<0.0001*
Species	0.786	3	3	3.673	0.157
Sampling date	1.043	6	8	1.453	0.305
Univariate analysis of site (Jacksonville, FL)					
	Type III SS	d.f.		*F*	*P*
Site					
PCoA1	0.409	4		10.417	<0.01*
PCoA2	0.189	4		4.752	<0.05*
PCoA3	0.461	4		48.283	<0.0001*
(C) Multivariate analysis of larval libraries from only multispecies sites					
Source of variation	Pillai's trace	Hypothesis d.f.	Error d.f.	*F*	*P*
Site	1.926	6	6	26.036	<0.001*
Species	0.804	6	6	0.672	0.679
Sampling date	0.847	9	12	0.525	0.831
Univariate analysis of site (multispecies)					
	Type III SS	d.f.		*F*	*P*
Site					
PCoA1	0.968	2		56.247	<0.0001*
PCoA2	0.718	2		44.357	<0.0001*
PCoA3	0.0535	2		10.745	<0.01*

P-values from MANOVA (multivariate) and ANOVA (univariate) are based on Bray-Curtis distances (*significant at the ≤ 0.05 level).

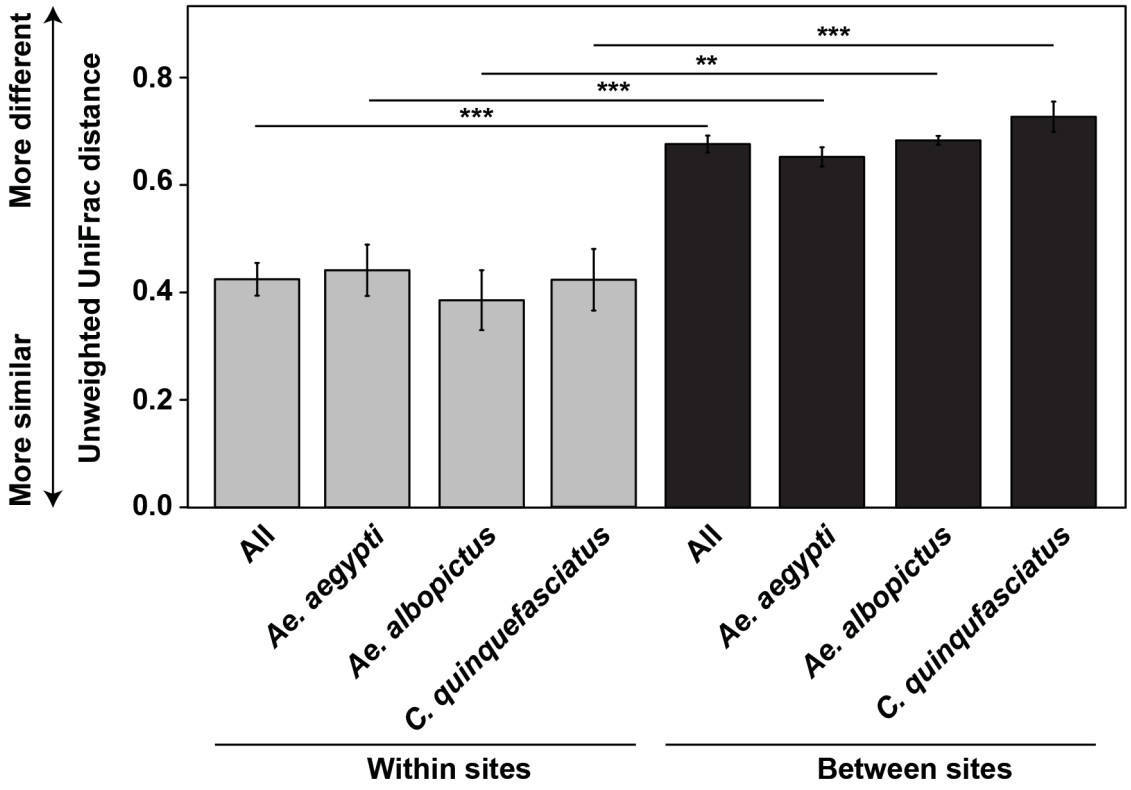


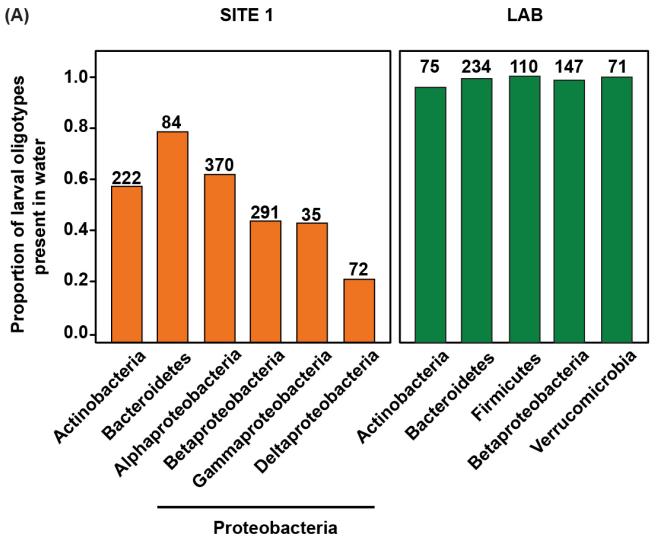
Figure 3.4. Within- and between-site variation in the bacteria in mosquito larvae. Within-site variation was determined by average unweighted UniFrac distances between different mosquito species from the same site, while between-site variation was determined by distances between the same mosquito species collected from different sites. Mean values ± 95% confidence intervals are shown. **, $P < 0.01$; ***, $P < 0.0001$ (Student's t-test with 10,000 Monte Carlo permutations).

Oligotyping identifies additional bacterial diversity

Oligotyping provides a computational method for identifying subpopulations (oligotypes) within OTUs. We therefore generated oligotyping data sets for the six most abundant bacterial phyla we identified (Proteobacteria, Actinobacteria, Bacteroidetes, Firmicutes, Cyanobacteria and Verrucomicrobia), which as noted above accounted for 81% of the OTU groups we identified across all samples. A total of 2.6 million quality-

filtered reads were used, which ranged from 3,301 to 164,841 per sample, and grouped into 4,321 unique oligotypes (Table B.5). Sequence identities within an oligotype ranged from 99.5% to 100%, while relative abundance of individual oligotypes ranged from 0.006% to 0.3%.

We used oligotyping to further assess bacterial diversity by focusing on two locations: Jacksonville field site 1, which on 21 July 2014 contained *Ae. aegypti* and *C. quinquefasciatus* larvae, and our laboratory culture of *Ae. aegypti,* which was sampled on a single date. We first determined the total number of oligotypes for each of the bacterial phyla or classes (Proteobacteria) that contained >5% of the OTU groups in *Ae. aegypti* larvae from these collections. For Jacksonville field site 1, these taxa were Actinobacteria, Bacteroidetes and four classes of Proteobacteria, while for the laboratory culture they were Actinobacteria, Bacteroidetes, Firmicutes, Betaproteobacteria and Verrucomicrobia (Figure 3.5A). We then plotted the proportion of these oligotypes that were present in water from the same site. A majority of the oligotypes detected in *Ae. aegypti* larvae from field site 1 were detected in the water for some taxa (Bacteroidetes) but not others (Deltaproteobacteria) (Figure 3.5A). In contrast, almost all oligotypes in *Ae. aegypti* larvae from the laboratory culture were detected in the water habitat (Figure 3.5A).



(B)

		Number of oligotypes in *Ae. aegypti*	SITE 1 (7/21/2014) *C. quinquefasciatus*	SITE 1 (7/21/2014) Water	SITE 3 (7/21/2014) *Ae. aegypti*	SITE 4 (7/21/2014) *Ae. aegypti*	LAB *Ae. aegypti*
	Actinobacteria *Leucobacter sp.*	32	32	14	1	0	0
	Bacteroidetes *Dysgonomonas sp.*	72	72	64	0	2	0
Proteobacteria	**Alphaproteobacteria** uncl. Alphaproteobacterium	370	322	229	6	17	3
Proteobacteria	**Betaproteobacteria** *C39 sp.*	76	73	74	1	1	0
Proteobacteria	**Gammaproteobacteria** uncl. Sinobacteraceae	5	5	5	0	0	0
Proteobacteria	**Deltaproteobacteria** *Desulfovibrio cuneatus*	72	72	15	0	0	0

		Number of oligotypes in *Ae. aegypti* (7/21/2014)	SITE 1 *Ae. aegypti* (9/15/2014)	SITE 1 Water (9/15/2014)
	Actinobacteria *Leucobacter sp.*	32	32	3
	Bacteroidetes *Dysgonomonas sp.*	72	72	46
Proteobacteria	**Alphaproteobacteria** uncl. Alphaproteobacterium	370	40	54
Proteobacteria	**Betaproteobacteria** *C39 sp.*	76	0	37
Proteobacteria	**Gammaproteobacteria** uncl. Sinobacteraceae	5	5	5
Proteobacteria	**Deltaproteobacteria** *Desulfovibrio cuneatus*	72	0	3

(C)

	Number of oligotypes in *Ae. aegypti*	LAB Water	SITE 1 (7/21/2014) *Ae. aegypti*	SITE 3 (7/21/2014) *Ae. aegypti*	SITE 4 (7/21/2014) *Ae. aegypti*
Actinobacteria uncl. Microbacteriaceae	72	72	64	0	0
Bacteroidetes *Chryseobacterium sp.*	35	35	0	5	0
Betaproteobacteria uncl. Comamonadaceae	145	144	1	0	0
Firmicutes *Clostridium sp.*	110	110	1	0	0
Verrucomicrobia *Luteolibacter* sp.	42	42	0	1	0

Figure 3.5. Oligotype analyses. (A) Oligotype overlap between *Aedes aegypti* larvae and water collected from site 1 in Jacksonville, FL (7/21/2014) or the laboratory. Bars indicate the proportion of oligotypes present in larvae that were also present in water. Numbers above the bars indicate the total number of oligotypes present in larvae. (B) Oligotype distribution between larvae collected from sites 1, 3 or 4 in Jacksonville, FL (7/21/2014) and the laboratory. The column on the left indicates the number of oligotypes present in *Ae. aegypti* larvae from site 1 whose taxonomic assignment matched the dominant OTU corresponding to a given phylum (or class). Subsequent bars indicate the proportion of these oligotypes present in either *Culex quinquefasciatus* larvae or water collected from the same site, or *Ae. aegypti* larvae collected from other sites on the same collection date. Bars on the far right indicate the proportion of these oligotypes present in *Ae. aegypti* larvae in the laboratory. The lower panel displays the proportion of oligotypes present in *Ae. aegypti* larvae from site 1 that were present in either *Ae. aegypti* larvae or water collected from the same site on 9/15/2014. Bars in (C) present oligotype overlap between *Ae. aegypti* larvae collected from the laboratory and the same field sites. The column on the left indicates the number of oligotypes present in *Ae. aegypti* larvae from the laboratory corresponding to the dominant taxa identified by OTU clustering. Subsequent bars indicate the proportion of these oligotypes present in either water collected from the same site, or *Ae. aegypti* larvae collected from sites 1, 3 or 4 in Jacksonville, FL (7/21/2014). Only phyla (or classes) that accounted for >5% of the total number of reads are included in the figure.

We next selected the most abundant OTU for each of the taxa shown in Figure 3.5A and determined the number of oligotypes for each in *Ae. aegypti* larvae. The taxonomic assignments for these OTUs are summarized in Table B.6, which also presents the sequences for the two most abundant oligotypes, and the full-length sequences for the corresponding 16S rRNA gene amplicons. We also separately examined the number and identity of the oligotypes for each of the OTUs in the three technical sequencing replicates for these samples. This showed that the number and identity of the oligotypes for each OTU per replicate were identical. Comparison of these OTUs

and oligotypes to the *C. quinquefasciatus* larvae that were also present at Jacksonville field site 1 on 21 July 2014 showed almost complete overlap, whereas only partial overlap was detected in the water (Figure 3.5B). Comparison to *Ae. aegypti* larvae collected from site 1 on 15 September 2014 also showed that some OTUs and corresponding oligotypes near fully overlapped (*Leucobacter*, *Dysgonomonas*), while others (C39, *Desulfovibrio cuneatus*) were absent although some oligotypes were detected in the water (Figure 3.5B). In contrast, almost no overlap in oligotypes was detected in *Ae. aegypti* larvae collected from Jacksonville field sites 3 and 4 on 21 July 2014 or the laboratory, because either these OTUs were absent or very rare (Figure 3.5B). The same exercise with the most abundant OTUs in *Ae. aegypti* larvae from the laboratory showed near complete overlap with the oligotypes detected in the water (Figure 3.5C). In contrast, little overlap was detected with *Ae. aegypti* larvae from different Jacksonville field sites (Figure 3.5C). The one exception to these trends was the unclassified Microbacteriaceae where a number of oligotypes overlapped with *Ae. aegypti* larvae from Jacksonville field site 1. However, this OTU was also very rare in site 1 larvae with almost all of the overlapping oligotypes detected as single sequences (singletons).

For most of the OTUs discussed above, the two most abundant oligotypes differed from each other by only 1 nucleotide with corresponding full-length amplicons

exhibiting ≥99% identity (Table B.6). Exceptions were the unnamed genus C39 (Betaproteobacteria) detected in *Ae. aegypti* larvae from Jacksonville field site 1, where the two most abundant oligotypes derived from full-length amplicons that differed by 29 nucleotides over 463 nt (92% sequence identity), and the *Chryseobacterium* (Bacteroidetes) in *Ae. aegypti* larvae from the laboratory, where the two most abundant oligotypes derived from full-length amplicons that differed by 96 nucleotides over the 453 nt (79% sequence identity). The strongest matches identified after BLASTN searches in most cases exhibited ~99% sequence identity with known bacteria (Table B.6). Most matches were to uncultured bacterial clones identified from different parts of the world in freshwater, rainwater or soil (Table B.6). Notably though, oligotype 1 for C39 shared 99% sequence identity with an uncultured bacterium identified in the larval gut of *Anopheles stephensi*, while oligotype 1 for the *Chryseobacterium* in laboratory *Ae. aegypti* larvae shared 99% sequence identity with an uncultured clone from a crane fly, *Tipula abdominalis*, which resides in the sister family (Tipulidae) of the Culicidae (Table B.6).

Mosquitoes from the field and laboratory both require gut bacteria for development

The preceding results indicated the microbiota in *Ae. aegypti*, *Ae. albopictus* and *C. quinquefasciatus* larvae differ between local field sites and collection dates as well as between field sites and the laboratory. We therefore asked whether these differences affected the requirement of mosquitoes from the field and laboratory for gut bacteria to develop. Examining progeny from every field site for these experiments was not technically feasible because of the effort involved in rearing field-collected mosquitoes, producing eggs and generating progeny with no gut bacteria. We therefore focused these experiments on comparing the laboratory population of *Ae. albopictus* to *Ae. albopictus* from the field site in Athens, GA, and *Ae. aegypti* plus *C. quinquefasciatus* from the laboratory to the same species collected at Jacksonville field site 1. These choices were justified because each of these populations differed in terms of deriving from sites with distinctly different bacterial communities, having different culture histories, and in the case of *Ae. aegypti* also differing in regard to *Wolbachia* infection. We first verified that previously developed methods for surface-sterilizing eggs (Coon et al. 2014) produced larvae with no gut bacteria. For each species, culture-based assays showed that numerous bacterial colonies grew when homogenates of first instars from non-sterilized eggs were plated on LB or BHI plates. PCR assays also generated amplicons using

universal 16S rRNA bacterial gene primers and DNA templates from non-sterilized eggs. In contrast, no bacteria grew when homogenates of first instars from surface-sterilized eggs were placed on culture plates. No bacterial amplicons were detected using universal primers and templates from laboratory (UGAL) *Ae. aegypti* first instars that hatched from surface-sterilized eggs but weak amplicons were detected in the other samples that we assumed were due to *Wolbachia*. This interpretation was supported using primers for the *Wolbachia wsp* gene, which generated amplicons from each sample except UGAL *Ae. aegypti*. Thus, surface sterilization of eggs produced axenic larvae with no detectable bacteria in the case of UGAL *Ae. aegypti*, whereas larvae for all other samples likely had no gut bacteria but, as expected, remained infected with *Wolbachia*, which are vertically transmitted and intracellular. We then placed larvae from surface-sterilized eggs in sterile water plus sterile food. All ultimately died as first instars after hatching (Figure 3.6). Larvae in this condition usually died 6–9 days after hatching, but some lived up to 14 days as first instars that never moulted before finally dying. In contrast, a majority of larvae from each collection site and species developed into adults if inoculated with water from laboratory trays containing conventionally reared (non-sterile) *Ae. aegypti* larvae or water from field site 1 in Jacksonville, FL (Figure 3.6). A majority of larvae from surface-sterilized eggs also developed into adults if inoculated

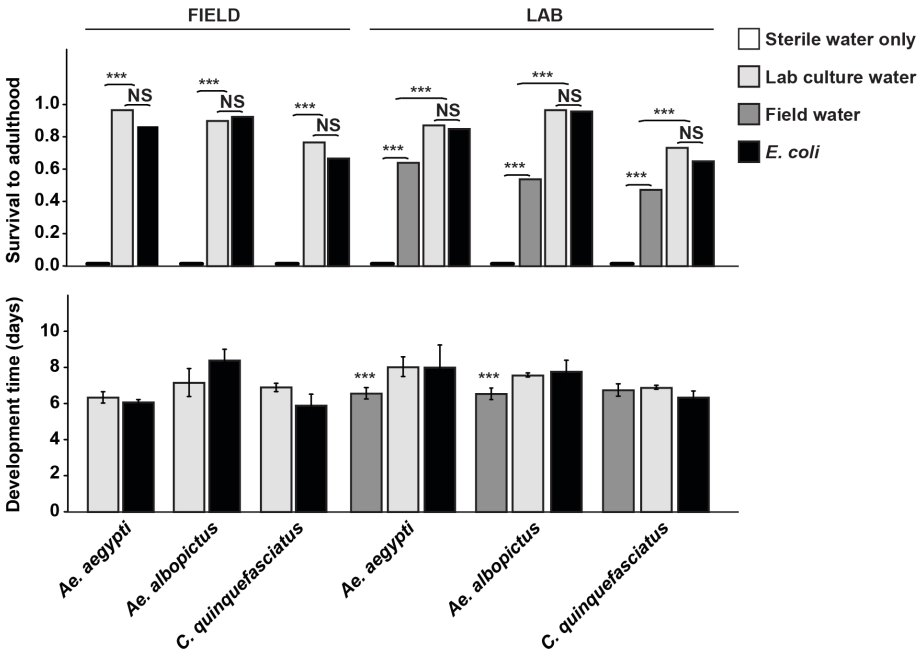


Figure 3.6. Survival to adulthood and development time of *Aedes aegypti*, *Aedes albopictus* and *Culex quinquefasciatus* larvae that hatched from eggs laid by adult, mated females reared from field-collected larvae or larvae from laboratory cultures. Field-derived *Ae. aegypti* and *C. quinquefasciatus* came from site 1 in Jacksonville, FL, while *Ae. albopictus* came from the field site in Athens, GA. All laboratory-reared females came from our long-term laboratory cultures (see 'Materials and Methods'). All eggs were surface-sterilized, and the first instars that hatched were confirmed to contain no gut bacteria. The bars in the upper graph show the proportion of larvae that developed into adults when fed sterilized diet in sterile water, sterile water inoculated with 100 µl of non-sterile laboratory water, sterile water inoculated with 100 µl of non-sterile field water and sterile water inoculated with *Escherichia coli*. Fisher's exact tests were conducted separately for each species with post hoc Bonferroni-corrected pairwise tests to compare treatments (NS, treatments not significantly different; ***, sterile water only treatment significantly differs from the laboratory culture water, field water and *E. coli* treatments, $P < 0.0001$). The bars of the lower graph show the development time from egg hatching until adult emergence for larvae reared in water inoculated with laboratory culture water, field water and *E. coli*. Development time for larvae cultured in sterile water only is not shown in the lower graph because no individuals survived beyond the first instar. Columns indicate mean values with 95% confidence intervals. ANOVA and post hoc comparison tests were used to compare development times separately for each species (*** laboratory-derived *Ae. aegypti* and *Ae. albopictus* reared in cultures inoculated with field water developed faster than larvae in cultures inoculated with laboratory water or *E. coli*, $P < 0.05$).

with only *Escherichia coli*, which was absent from all field and laboratory samples we analysed (Figure 3.6).

Antibiotics do not fully eliminate bacteria from conventionally reared *Aedes aegypti* larvae and adversely affect axenic larvae that contain no bacteria

The preceding results indicated that *Ae. aegypti*, *Ae. albopictus* and *C. quinquefasciatus* require gut bacteria for development regardless of origin, prior rearing history or *Wolbachia* infection. These results were also near identical to our previous results that examined only long-term laboratory cultures of *Ae. aegypti*, *Ae. atropalpus* and *An. gambiae* that were not *Wolbachia* infected (Coon et al. 2014). However, they clearly differed from results showing that mosquito larvae treated with antibiotics only exhibit delays in development (Chouaia et al. 2012). We thus assessed whether antibiotic treatment results in developmental delays because bacteria that colonize mosquitoes are not fully eliminated. As with our developmental assays, it was not logistically feasible to examine the antibiotic susceptibility of every OTU we identified across the species and breeding sites we sampled. We therefore selected seven abundant community members that we isolated from UGAL *Ae. aegypti* and field-collected *Ae. albopictus*. Like *E. coli*, each individually colonizes *Ae. aegypti* and *Ae. albopictus* from surface-sterilized eggs to

produce gnotobiotic larvae that develop normally into adults. We first tested the susceptibility of these bacteria to several different antibiotics over a concentration range used in other studies to eliminate bacteria from the mosquito digestive tract (Dong et al. 2009; Chouaia et al. 2012; Ramirez et al. 2012; Minard et al. 2013). The purpose of this was to assess the sensitivity of each bacterium to these antibiotics in the absence of any confounding effects from being in a mosquito. Results showed that these bacteria exhibited highly variable sensitivities with the minimal inhibitory concentration (MIC) for ampicillin exceeding 200 μg/ml for each species while MICs for kanamycin, streptomycin and penicillin exceeded 200 μg/ml for most species (Table B.7). Average MICs were lower for tetracycline, chloramphenicol and gentamicin but were ≥200 μg/ml for a *Chryseobacterium* sp. isolated from UGAL *Ae. aegypti* (Table B.7). Thus, *in vitro* each bacterium showed differential sensitivity to the antibiotics we tested and in some cases were largely unaffected at high doses.

Given these findings, we next assessed the effects of each antibiotic on larvae across the same range of concentrations. Given the highly variable microbial communities we identified across species and populations but the inability of each to develop without gut bacteria, we selected conventionally reared (non-sterile) UGAL *Ae. aegypti* for these assays, which was also the source of most bacterial isolates for the preceding MIC assays. Results indicated these larvae moulted to the second instar in 2

days in the absence of antibiotics and across all concentrations of ampicillin (Figure 3.7A). The other antibiotics had dose-dependent effects on mortality, timing of moulting or both (Figure 3.7A). Chloramphenicol and penicillin had no mortality effects but all larvae moulted to the second instar within 5 days. All or a large proportion of larvae treated with 200 μg/ml of kanamycin, streptomycin or tetracycline or ≥20 μg/ml of gentamicin died within 5 days, but all surviving larvae moulted within 5 days (Figure 3.7A). Larvae treated with lower concentrations of these antibiotics exhibited no mortality and moulted within 2 days (Figure 3.7A).

To quantify bacterial clearance, individual larvae from cultures containing the highest concentration of each antibiotic that did not cause larvae to die were homogenized at 2 days post-hatching and cultured on LB plates. Results showed that each antibiotic except ampicillin significantly reduced the number of culturable bacteria when compared to conventional larvae that were cultured with no antibiotic (Figure 3.7B). However, all antibiotic treated larvae still contained viable bacteria, whereas axenic larvae produced from surface-sterilized eggs contained, as expected, no culturable bacteria (Figure 3.7B). We therefore assessed whether the mortality kanamycin, streptomycin, tetracycline and gentamicin caused in conventional larvae when used at 200 μg/ml also occurred in axenic larvae. Dose–response assays showed that these antibiotics as well as kanamycin and streptomycin dose-dependently increased the

mortality of axenic larvae when compared to axenic control larvae that were cultured in the absence of antibiotics (Figure 3.7C). Altogether, these results indicated that several antibiotics failed to fully eliminate bacteria from UGAL *Ae. aegypti* while also showing that antibiotic treatment adversely affects first instars when used at high concentrations regardless of whether or not they contain culturable bacteria.

(A)

Antibiotic		N	Mortality	Moulting after 2 d	Moulting after 5 d
Ampicillin					
	200 µg/ml	48	0	48	-
	20 µg/ml	48	0	48	-
	2 µg/ml	24	0	24	-
	0 µg/ml	24	0	24	-
Kanamycin					
	200 µg/ml	72	41	2*	29
	20 µg/ml	72	0	33*	39
	2 µg/ml	48	0	48	-
	0 µg/ml	48	0	48	-
Streptomycin					
	200 µg/ml	72	21	3*	48
	20 µg/ml	72	0	37*	35
	2 µg/ml	48	0	48	-
	0 µg/ml	48	0	48	-
Chloramphenicol					
	200 µg/ml	48	0	24	24
	20 µg/ml	48	0	26	22
	2 µg/ml	24	0	24	-
	0 µg/ml	24	0	24	-
Penicillin					
	200 µg/ml	72	0	15*	57
	20 µg/ml	72	0	48	24
	2 µg/ml	48	0	48	-
	0 µg/ml	48	0	48	-
Tetracycline					
	200 µg/ml	48	48	-	-
	20 µg/ml	48	0	45	3
	2 µg/ml	24	0	24	-
	0 µg/ml	24	0	24	-
Gentamicin					
	200 µg/ml	72	68	1*	3
	20 µg/ml	72	47	14*	11
	2 µg/ml	72	0	72	-
	0 µg/ml	48	0	48	-

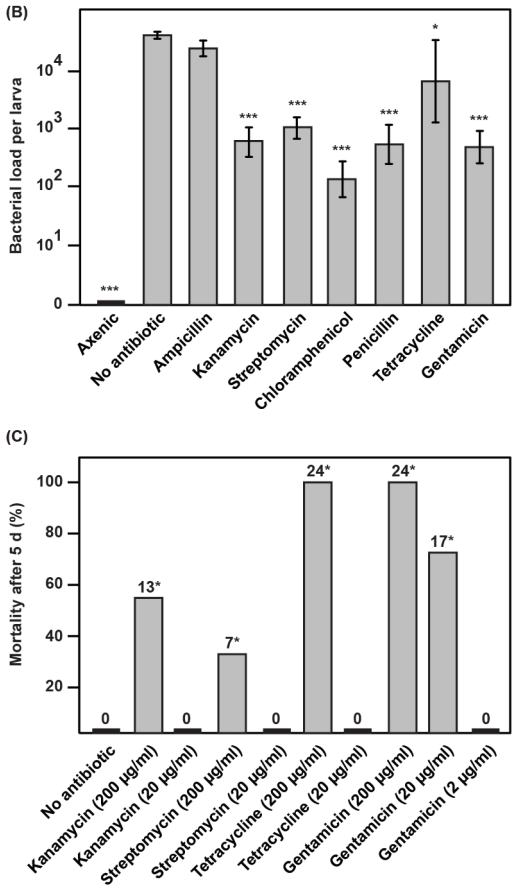


Figure 3.7. Effect of antibiotics on UGAL strain *Aedes aegypti* larvae and associated bacteria. (A) Mortality and moulting of conventionally reared (non-sterile) first instars treated with different antibiotics that were added to cultures. Columns from left to right indicate the following: antibiotic type, dose, total number of larvae treated per dose, the number of larvae that died within 5 days, the number of larvae that moulted after 2 days and the number of larvae that moulted after 5 days. Asterisks (*) in the column labelled 'Moulting after 2 d' indicate the treatment significantly differed from the negative control, which was larvae treated with no antibiotic (0 μg/ml) ($P < 0.05$; Fisher's exact test, pairwise comparison against control). (B) Bacterial loads in 2-day-old larvae collected from treatment dishes as described in (A). Non-sterile first instars reared conventionally without antibiotics served as the positive control. First instars from surface-sterilized eggs reared under sterile conditions (Axenic) served as the negative control. In most cases, a total of six larvae were sampled from the highest dose for each antibiotic treatment (200 μg/ml). In the case of tetracycline and gentamicin, larvae were sampled from the 20 μg/ml treatment due to 100% mortality at 200 μg/ml (see A). Bars show mean bacterial load per larva with 95% confidence intervals as determined by colony-forming units. An asterisk (*) above a bar indicates the treatment significantly differed from the positive control (*, $P < 0.05$; ***, $P < 0.0001$; Dunnett's test). (C) Mortality of axenic first instars reared in the presence of antibiotic. Antibiotic treatment and corresponding dose are indicated below each bar. Axenic first instars reared under sterile conditions without antibiotic treatment served as the control. A total of 24 larvae were assayed per treatment. Numbers above the bars indicate the number of larvae that died within 5 days, while an asterisk (*) following this number indicates significantly more larvae died relative to the control ($P < 0.05$; Fisher's exact test, pairwise comparison against control).

3.5 Discussion

Our previous results (Coon et al. 2014; 2016a) indicated that laboratory populations of three mosquito species required living bacteria to develop into adults, but development did not depend on a particular bacterial species or community assemblage. In this study, we generated additional information in three areas of study to better understand the functional role of bacteria in mosquito development. This included the characterization

of bacterial community diversity within and between three species from breeding sites in the field and laboratory. This information was essential to know whether or not species or larvae from different locations host similar or different bacterial communities of potential importance in development. With this knowledge in hand, we then could assess whether (i) larvae differ in their requirement for living bacteria to develop as a function of species, rearing history, overall bacterial community composition or (ii) Wolbachia infection that has previously been suggested to nutritionally benefit mosquitoes during periods of stress (Brownlie et al. 2009). These data also fully positioned us to assess whether the approach of producing axenic larvae by surface-sterilizing eggs versus treating larvae with antibiotics yields similar or different outcomes.

Our analyses of bacterial community diversity indicate that *Aedes aegypti*, *Aedes albopictus* and *Culex quinquefasciatus* larvae primarily contain a subset of the OTUs in the water they were collected from, which supports that larvae are colonized by a subset of the bacteria they ingest during feeding. As our data derive from pooled samples of whole larvae, we cannot conclude that the OTUs we identified are all restricted to the gut. However, prior results as well as data in this study showing that axenic larvae contain no bacteria unless infected by *Wolbachia* strongly support that larvae acquired the bacteria we identified from the environment by feeding which in turn also suggests

most of the OTUs we identified are present in the digestive tract. However, it is also possible some bacteria present in the gut over time colonize other tissues given findings in the literature that identify some species of bacteria present in the gut that also colonize other organs (see Chouaia et al. 2012; Gimonneau et al. 2014).

We recognize that additional sampling could further improve upon our bacterial diversity studies. However, our data set was sufficiently robust to compare within-site versus between-site beta diversity, which strongly supported that community composition varied greatly between sites, while strong similarities were found between larvae of different species that were developing in the same site. That no species effect was detected in sites containing two species of mosquitoes supports that a similar subset of bacteria colonized larvae of each. That identical oligotypes were recovered from water and larvae within but not between sites is additional strong evidence for between-site variability for the bacterial communities in water and larvae. Interestingly, our within-site comparisons between different sampling dates also indicate that the bacterial communities in larvae and their aquatic habitats become more similar over the breeding season. At this time, we are uncertain what accounts for this. Certainly, bacteria from diverse sources could colonize the sites we sampled. However, it is also possible that mosquitoes modify their aquatic habitat through cyclic transmission, which has been proposed for other aquatic organisms (McFall-Ngai 1998; Sullam et al. 2012). This could

occur through larvae feeding and excreting bacteria that are selected for in their own digestive tract. We also previously showed that some abundant members of the larval and adult gut communities in laboratory-reared *Ae. aegypti* are present on the surface of eggs females lay (Coon et al. 2014). Thus, adult mosquitoes may transmit some gut community members to habitats where they oviposit, which over time could affect the microbial community in the water where larvae develop. The ability of mosquitoes to alter the composition of bacterial communities in larval habitats would be strongest in small volume containers like those we sampled. Comparison of sites persistently colonized by mosquito larvae to neighbouring sites where no mosquitoes can colonize would be one approach to assessing whether mosquitoes significantly alter the bacterial communities in small aquatic habitats.

Our community diversity data share some parallels with studies of terrestrial insects including *Drosophila* and certain Lepidoptera, which show that the food larvae consume overrides the effect of species in defining the composition of the gut microbiota (Chandler et al. 2011; Wong et al. 2011; Priya et al. 2012). However, it has also been noted that the complex relationships between bacteria and their environments may require finer-scale information about microbial diversity than can be provided by the classification of OTUs or clustering approaches (Eren et al. 2013). Our use of oligotyping allowed us to address this concern and assess whether concealed diversity

within identified OTUs reveals additional patterns. We generated oligotyping data sets for the six most abundant bacterial phyla detected across our entire sequencing data set. However, we focused on Jacksonville field site 1, which contained both *Ae. aegypti* and *C. quinquefasciatus* larvae, and our *Ae. aegypti* laboratory culture to assess whether oligotyping revealed similar or different trends to our clustering analyses. In terms of colonization, almost all oligotypes in laboratory-reared *Ae. aegypti* were present in the water they were collected from. This was also true for some bacterial taxa in *Ae. aegypti* from field site 1, but for others like Deltaproteobacteria, most of the oligotypes detected in larvae were not detected in the water they came from. More interesting though were our results that focused on the most abundant OTUs for each oligotyped phylum or class in *Ae. aegypti* larvae from Jacksonville field site 1. These data showed that each OTU consisted of several oligotypes but nearly all oligotypes detected in *Ae. aegypti* larvae were also present in *C. quinquefasciatus* larvae from the same site and date. This provided strong evidence that the same subset of bacteria in the local habitat colonized both species. In contrast, almost none of these OTUs or oligotypes were detected in *Ae. aegypti* larvae collected on the same date from other sites in Jacksonville or the laboratory, which underscores the high variation that existed between the sites we sampled.

As previously noted, the presence of *Wolbachia* in *Ae. albopictus* and *C. quinquefasciatus* was expected, but identification of A and B group *Wolbachia* in *Ae. aegypti* from Jacksonville site 1 was not because no natural infections of this species have previously been reported (Ross et al. 2016). We thus were very careful to document that these mosquitoes were correctly identified and that we genotyped the *Wolbachia* strains. We sequenced only a small number of adults from this site because we did not anticipate encountering any *Wolbachia* in *Ae. aegypti*, and characterizing these bacteria was not an original goal of the study. As a result, we had only a small number of frozen adults from this site that could be used for sequence analysis when we learned from the larval data set that *Wolbachia* reads were present. Nonetheless, the data we generated confirmed each adult contained the same *Wolbachia* OTUs detected in larvae. At this time, we need to be very cautious about these results and their possible implications because our data also clearly indicate that *Ae. aegypti* at most of the sites we sampled contained no *Wolbachia*. That both the A and B group members detected in *Ae. aegypti* from Jacksonville, FL, field site 1 are related but not identical to *Wolbachia* that have been identified from *Ae. albopictus* suggests the possibility of past lateral transfer, which would also be consistent with *Ae. aegypti* and *Ae. albopictus* sharing partially overlapping ranges in several parts of the world including the southeastern United States. The presence of a natural *Wolbachia* infection in *Ae. aegypti*

also has applied implications given the use of introduced *Wolbachia* in *Ae. aegypti* for use in population replacement to alter vector competency (reviewed in Minard et al. 2013).

While *Wolbachia* abundance in larval stage mosquitoes has not previously been reported, our finding that titres are very low relative to adults is similar to a recent study, which noted that *D. melanogaster* larvae also contain a low abundance infection relative to adult females (Stevanovic et al. 2015). On the other hand, the high abundance of *Wolbachia* in adult female mosquitoes has been reported in other studies and has also been noted to hinder characterization of gut microbiota diversity in pyrosequencing data sets (Minard et al. 2014; Muturi et al. 2016). Our Illumina data in contrast provided sufficient coverage to discern that adults contained a subset of the OTUs detected in larvae, which circumstantially supports results from previous laboratory experiments that adults acquire a subset of the gut community in larvae by transstadial transmission (Coon et al. 2014).

Collectively, our bacterial diversity and *Wolbachia* data positioned us to address the second goal of this study by assessing whether larvae differed in their developmental requirement for gut bacteria as a function of species, origin (laboratory versus field) or *Wolbachia* infection status. Results showed they did not with all larvae failing to develop when gut bacteria were absent but a high proportion of larvae developing into

adults when recolonized with a mixed community of bacteria in water from the field or laboratory, or *Escherichia coli*, which was absent across all samples and collection sites. In contrast, the third goal of our study revealed two important outcomes in regard to antibiotics. First, while antibiotics are commonly used to eliminate resident microbiota from insects including mosquitoes, our results indicate they do not fully eliminate the microbiota from *Ae. aegypti* larvae, which is one reason why antibiotic therapy does not prevent larvae from developing. A recent study similarly revealed that antibiotic treatment also does not fully eliminate the microbiota from adult *Anopheles* mosquitoes (Hughes et al. 2014). Second, some of the antibiotics we tested adversely affected the survival of *Ae. aegypti* which contained no bacteria. While no published work has previously noted antibiotic sensitivity in mosquito larvae, some antibiotics including tetracyclines are known to adversely affect other eukaryotes including insects independent of their effects on the microbiota (Ridley et al. 2013; Moullan et al. 2015). We thus conclude that surface sterilization of eggs is much more effective for producing mosquito larvae with no gut bacteria and that antibiotic therapy has several major shortcomings for studying the function of bacteria in mosquito development.

We have now examined a total of five mosquito species from field and laboratory populations that each exhibit a requirement for living bacteria in their gut to develop, but also show no evidence that development depends on a particular species or

community assemblage. While several studies have noted variable communities of bacteria in mosquitoes from different geographic locations, this study shows that bacteria are consistently present in mosquito larvae but community composition is also highly variable between sites on a fine geographic scale. Together, these data suggest the hypothesis that the mosquito species we have studied to date rely on bacteria for development quite generally because of the high unpredictability in the community of bacteria between sites. This lack of specialization could also reflect that most of the mosquitoes we have studied have been introduced into habitats outside of their native range. More specialized mosquito species or these species in their native habitat in contrast may exhibit more specific associations with bacteria.

The obvious question going forward is what do living bacteria provide that mosquito larvae require? This issue is also very interesting from the perspective of the model organism literature where *Drosophila* and mice show roles for gut bacteria in maturation of the immune and digestive systems but also indicate bacteria are not required for development into adults because axenic cultures of both can be maintained for generations if fed a nutritionally complete diet (Buchon et al. 2013; Lee & Brey 2013; Sommer & Backhed 2013; Blaser 2016; Goodrich et al. 2016). One option is that many bacteria share conserved metabolic features that facilitate breakdown of factors in the larval diet or could produce nutrients that supplement the mosquito diet. Several

studies have experimentally demonstrated or provide circumstantial support for gut bacteria having digestive or nutritional functions in some insects (summarized by Engel & Moran 2013). However, most of these examples focus on species that feed on specialized diets with nutrient constraints, whereas laboratory-reared mosquitoes consume nutritionally rich and complete diets. Alternatively, several different types of bacteria could potentially affect physiochemical parameters such as pH or redox state that alter the gut environment in ways that mosquito larvae depend upon to grow and moult. A third option is that most bacteria share cell wall components and/or generate conserved metabolites that could function as signalling molecules. The former are well-known pattern recognition factors that the insect immune system recognizes, which could activate different signalling pathways in the gut that are essential for development. Contact-dependent interactions between bacteria and the gut epithelium or diffusible bacterial metabolites that gut cells perceive have also been identified to mediate gut homeostasis or systemic growth of mice and *Drosophila* (Shin et al. 2011; Storelli et al. 2011; Nicholson et al. 2012). Understanding the mechanism(s) underlying why mosquitoes require living bacteria is of translational significance, because the processes involved may be amenable to disruption, which could be used as a tool for controlling species that transmit human disease.

3.6 Acknowledgements

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CHAPTER 4

BACTERIA-MEDIATED HYPOXIA FUNCTIONS AS AN ESSENTIAL SIGNAL FOR MOSQUITO DEVELOPMENT[3]

[3]Coon, K.L., Valzania, L., Brown, M.R. and M.R. Strand. Submitted to *Proceedings of the National Academy of Sciences USA*, 02/21/2017.

4.1 Abstract

Mosquitoes host communities of microbes in their digestive tract that consist primarily of bacteria. We previously reported that several mosquito species including *Aedes aegypti* do not develop beyond the first instar when fed a nutritionally complete diet in the absence of a gut microbiota. In contrast, several species of bacteria, including *Escherichia coli* K-12, rescue development of axenic larvae into adults. The molecular mechanisms underlying bacteria-dependent growth, however, are unknown. Here we designed a genetic screen around *E. coli* that identified high affinity cytochrome *bd* oxidase as an essential bacterial gene product for mosquito growth. Bioassays showed that bacteria in non-sterile larvae and gnotobiotic larvae inoculated with wild-type *E. coli* reduced midgut oxygen levels below 5%, whereas larvae inoculated with *E. coli* mutants defective for cytochrome *bd* oxidase did not. Experiments further supported that hypoxia signals through a hypoxia transcriptional program that leads to ecdysone-induced molting. Altogether, our results identify bacteria-induced gut hypoxia as a previously unknown but essential cue for mosquito development.

Keywords: insect, *Aedes aegypti,* microbiota, cytochrome *bd* oxidase, respiration, molting

4.2 Significance

Mosquitoes are important insects because several species transmit pathogens as adults that cause diseases in humans and other vertebrates. A promising approach for control is preventing immature mosquitoes from developing into adults. Immature stage mosquitoes require gut bacteria to develop but the mechanisms underlying this dependence are unknown. Here we identify cytochrome *bd* oxidase as a bacterial gene product that is essential for mosquito development. We also show that cytochrome *bd* oxidase lowers oxygen levels in the digestive tract of larvae, which serves as a signal for molting. These findings provide the first evidence that mosquito development relies on bacteria-dependent alterations in gut oxygen levels. This information can also potentially be used to develop tools for disabling mosquito development.

4.3 Introduction

Gut dwelling microbes are of interest because of their potential effects on growth, development and survival of animal hosts (Engel & Moran 2013; Sommer & Backhed 2013). Mosquitoes are insects of interest because many species transmit pathogens that cause disease in humans and other vertebrates. All mosquitoes are aquatic during their juvenile stages with larvae feeding primarily on detritus and small organisms (Merritt et al. 1992). As adults, most female mosquitoes blood feed on vertebrates, which creates

opportunities for transmission of pathogens between hosts (Clements 1992). Mosquitoes contain no microbes in their digestive tract after hatching from eggs, which results in larvae acquiring a gut microbiota anew each generation from the environment (Minard et al. 2013; Strand 2017). The majority of these community members are gram-negative aerobic and facultatively anaerobic bacteria (Wang et al. 2011; Boissiere et al. 2012; Coon et al. 2014; Gimonneau et al. 2014; Duguma et al. 2015; Muturi et al. 2016). Some but not all community members are also transstadially transmitted to adults (Coon et al. 2014; Duguma et al. 2015). Prior studies show that gut microbes in adult females can affect vector competence and pathogen transmission to vertebrates (Hegde et al. 2015; Van Tol & Dimopoulos 2016). They also indicate gut bacteria strongly affect development (Strand 2017). The absence of a tractable genetic model, however, has limited understanding of the molecular interactions that underlie bacteria-dependent growth of larvae into adults.

We recently established *Escherichia coli* K-12 BW25113 as a model for this purpose in conjunction with the mosquito *Aedes aegypti*. UGAL strain *Ae. aegypti* larvae reared under conventional (non-sterile) laboratory conditions contain a relatively simple community of ~100 bacterial species if fed a nutritionally complete diet (Coon et al. 2014). Larvae also molt through four instars before pupating and emerging as adults (Vogel et al. 2017). Axenic larvae with no gut microbiota die as first instars without

molting, but are rescued if inoculated with the community of bacteria present in a conventional culture (Coon et al. 2014). Several bacteria from this community and *E. coli* K-12, which is not a community member, also individually colonize the gut to produce gnotobiotic larvae that exhibit no differences in development time, adult size or fecundity relative to conventionally reared larvae (Coon et al. 2014; 2016a). Field collected *Ae. aegypti* and several other species of mosquitoes contain communities of gut bacteria that differ from laboratory reared *Ae. aegypti* but exhibit the same defects under axenic conditions as UGAL *Ae. aegypti* (Coon et al. 2014; Coon et al. 2016b). Their development is also rescued if inoculated with only *E. coli* (Coon et al. 2014; Coon et al. 2016b).

Taken together, several mosquito species die as first instars when fed a nutritionally complete diet in the absence of a gut microbiota, but develop into adults if living bacteria are present. Prior findings, however, argue against bacteria being a food source or provisioning nutrients essential for growth because axenic larvae do not develop beyond the first instar if fed dead bacteria, standard diet with dead bacteria, or standard diet that has been pre-conditioned by living bacteria before feeding (Coon et al. 2014). They also argue against *Ae. aegypti* requiring a particular species or community of bacteria because gnotobiotic larvae develop normally when singly colonized by several species including *E. coli* (Coon et al. 2014; 2016a,b). In this study,

we used *E. coli* to gain insights into what bacteria provide that *Ae. aegypti* requires. Our results identified cytochrome *bd* oxidase as an essential gene product. Functional data further indicated that bacteria reduce gut oxygen levels, which serves as an essential signal for growth and molting of all instars.

4.4 Results

Screening an *Escherichia coli* single-gene knockout library identifies several mutants that adversely affect larval growth

Within each instar, insect larvae grow by feeding until they achieve a critical size (Nijhout et al. 2013). Endocrine events associated with critical size then stimulate the release of the steroid hormone ecdysone, which induces molting. Under standardized conditions, conventionally reared *Aedes aegypti* larvae and gnotobiotic larvae inoculated with wild-type *Escherichia coli* K-12 achieve critical size (16 h post-hatching) and molt to the second instar (24 h post-hatching) at identical times (Vogel et al. 2017). Conventional and gnotobiotic larvae also synchronously molt to the third and fourth instar at 48 and 72 h post-hatching respectively. In contrast, axenic first instars feed but never reach critical size and die without molting (Vogel et al. 2017). We used this information and an *E. coli* K-12 knockout library of 3,985 single genes (Keio collection)

(Baba et al. 2006) in a forward genetic screen to identify mutants defective in stimulating molting. After inoculating newly hatched axenic larvae with each mutant, the vast majority (97%) had little or no adverse effect with most larvae molting by 24 h and all larvae molting by 48 h. However, we classified 131 mutants as defective because larvae either required longer than 48 h to molt or never molted.

We subclassified 48 of these mutants as growth defective because plate assays indicated that no bacteria were detected 24 h post-inoculation in the culture environment (Table C.1). We subclassified 79 others as colonization defective because viable bacteria were detected in the culture environment at 24 h post-inoculation but were not detected in homogenates of larvae (Table C.1). This suggested defects in the ability of these mutants to survive in the gut, which resulted in few or no larvae molting within 48 h. However, we determined for many of these mutants that the proportion of larvae molting within 48 h increased if cultures were inoculated with a larger number of bacteria (Table C.1). This indicated that defects were conditional with starting abundance likely affecting the number of viable bacteria that persisted in the culture environment and/or the larval gut. We also noted that a large proportion of the growth and colonization defective mutants lacked genes with roles in four broad gene ontology (GO) categories: central metabolism, amino acid biosynthesis/transport, peptidoglycan

recycling, or stress responses that could adversely affect bacterial growth under the culture conditions used to rear mosquitoes (Figure 4.1A; Table C.1).

The last 4 defective mutants (Δ*arcA*::*kan*, Δ*fnr*::*kan*, Δ*cydB*::*kan*, Δ*cydD*::*kan*) were of greatest interest because each was similarly abundant in cultures and first instars as wild-type *E. coli* at 24 h post-inoculation but the proportion of first instars

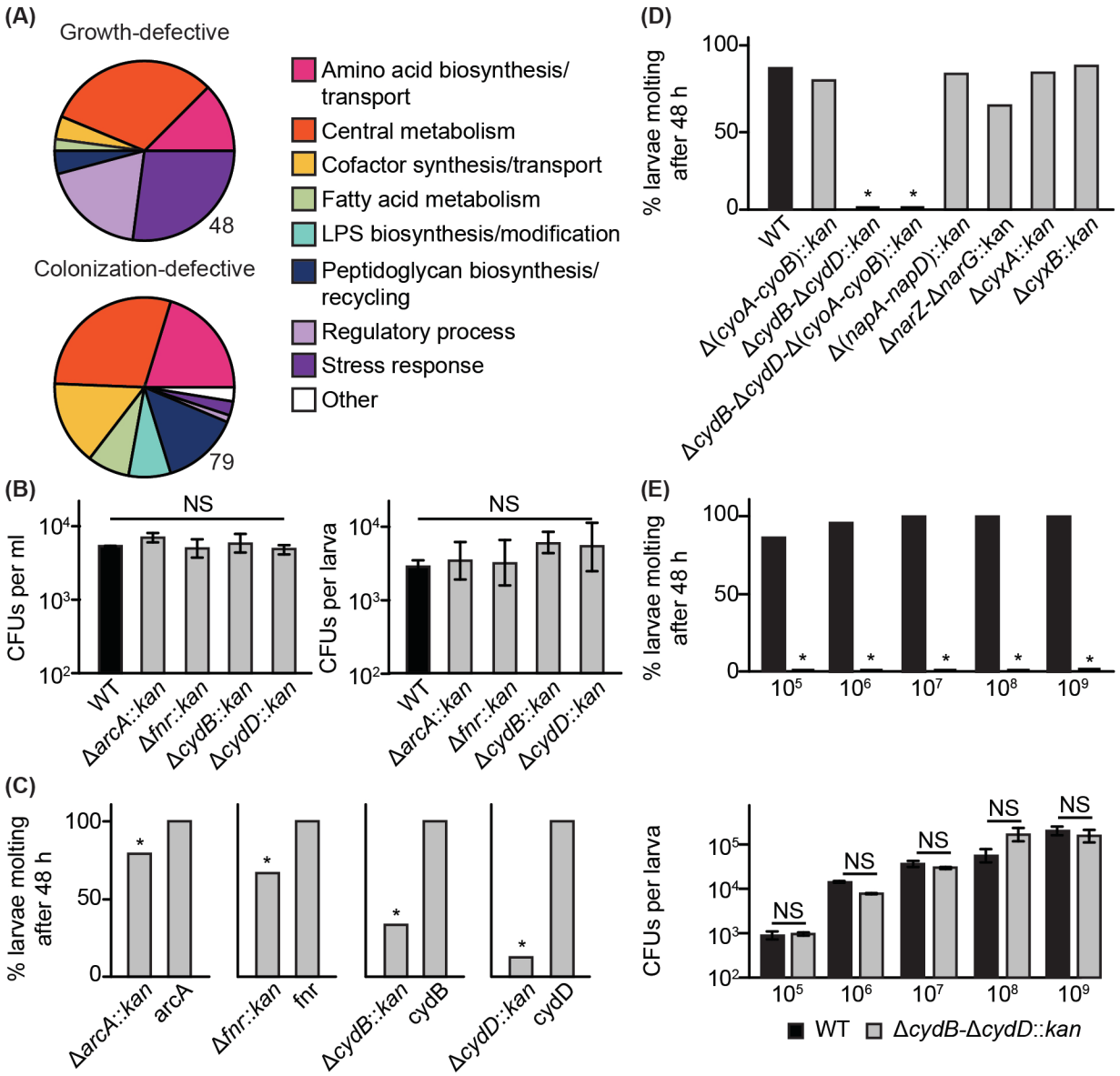


Figure 4.1. Multiple *Escherichia coli* mutants affect growth and molting of *Aedes aegypti* larvae. (A) Functional clustering of the 48 growth and 79 colonization defective single-gene deletion mutants. Pie charts show the GO categories to which deleted genes in the mutants belonged. Genes that fell into multiple GO categories are grouped together in the category designated as "Other". (B) Abundance of the four rescue defective mutants and wild-type (WT) *E. coli* in cultures (left panel) and larvae (right panel) at 24 h post-inoculation. A minimum of 4 replicates was assayed per treatment. Each bar indicates mean ± SE colony-forming units (CFUs). ANOVA detected no differences between treatments for either water ($F_{(4,14)} = 1.385$, $P = 0.289$) or larvae ($F_{(4,14)} = 1.536$, $P = 0.245$). (C) Percentage of first instars that molted when inoculated with each rescue defective mutant versus the same mutant transformed with an expression plasmid containing the deleted gene. An asterisk (*) indicates molting significantly differed between the two treatments with all rescued mutants stimulating 100% of larvae to molt ($P < 0.05$, Fisher's exact test). At least 24 larvae were assayed per treatment. (D) Percentage of first instar larvae molting to the second instar when inoculated with WT *E. coli* or the mutants Δ(*cyoA*-*cyoB*)::*kan*, Δ*cydB*-Δ*cydD*::*kan*, Δ*cydB*-Δ*cydD*-Δ(*cyoA*-*cyoB*)::*kan*, Δ(*napA*-*napD*)::*kan*, Δ*narZ*-Δ*narG*::*kan*, Δ*cyxA*::*kan*, and Δ*cyxB*::*kan*. * indicates a significant difference for a given mutant relative to the WT positive control ($P < 0.0001$, Fisher's exact test). At least 72 larvae were assayed per treatment. (E) The upper panel presents the percentage of first instars molting to the second instar when cultures were inoculated with 10^5-10^9 CFUs of WT versus Δ*cydB*-Δ*cydD*::*kan* *E. coli*. * indicates a significant difference between treatments ($P < 0.0001$, Fisher's exact test). At least 72 larvae were assayed per treatment. The lower panel presents the mean number ± SE of CFUs present per larva at 24 h post-inoculation for the cultures shown in the upper panel. For each inoculation amount, t-tests detected no significant difference (NS) in CFUs per larva between treatments ($P > 0.05$). A minimum of 4 individuals was assayed per treatment.

that molted by 48 h was significantly lower than for wild-type *E. coli* (Figure 4.1B,C; Table C.1). We subclassified these mutants as rescue defective. Notably, each had functions in respiration with *cydB* and *cydD* encoding products required for assembly of cytochrome *bd* oxidase, a terminal enzyme in the aerobic electron transport chain (Green & Gennis 1983; Poole et al. 1994), and *arcA* and *fnr* encoding regulators that

mediate expression of many genes including *cydB* and *cydD* that respond to aerobic versus anaerobic conditions (Lynch & Lin 1996).

The cytochrome *bd* oxidase respiratory pathway is essential for larval growth and molting

We assessed whether the molting defects associated with Δ*arcA*::*kan*, Δ*fnr*::*kan*, Δ*cydB*::*kan*, and Δ*cydD*::*kan* were rescued by *in trans* provision of each gene on a plasmid. Results indicated they were, which further suggested a role for *E. coli* respiration in larval growth and molting (Figure 4.1C). However, regulators like ArcA and Fnr mediate expression of other terminal oxidoreductases besides cytochrome *bd* oxidase, which together provide *E. coli* with respiratory flexibility as a facultative anaerobe. Under ambient (normoxic) aerobic conditions (21% O_2), *E. coli* predominantly uses low-affinity cytochrome bo_3 oxidase encoded by the *cyoABCDE* operon (Cotter & Gunsalus 1992). However, under lower (hypoxic) oxygen conditions (10-3% O_2), *E. coli* shifts to high-affinity cyctochrome *bd* oxidase produced from the *cydAB* and *cydDC* genes (Fu et al. 1991; Cotter & Gunsalus 1992). A third enzyme, cytochrome *bd* oxidase II (encoded by *cyxAB*) has also been suggested but not demonstrated to function under very low oxygen conditions (Brondsted & Atlung 1996). In contrast, anaerobic respiration in the vertebrate gut involves the reduction of nitrate by primary nitrate

reductase encoded by the *narGHJI* operon, secondary nitrate reductase encoded by the *narZYWV* operon, and periplasmic nitrate reductase encoded by the *napFDAGHBC* operon (Stewart 1993; Stewart et al. 2002; Tiso & Schechter 2015; Rivera-Chavez et al. 2016).

We therefore conducted a systematic mutational analysis to determine whether more than one *E. coli* respiratory pathway affected growth and molting of *Ae. aegypti* first instars. For aerobic respiration we produced a double mutant that deleted the genes encoding subunits I and II of cytochrome bo_3 oxidase (Δ(*cyoA*-*cyoB*)::*kan*), a double mutant that deleted the gene for subunit II of cytochrome *bd* oxidase and the gene for a transporter subunit required for assembly in the membrane (Δ*cydB*-Δ*cydD*::*kan*) (Green & Gennis 1983; Poole et al. 1994), and a quadruple mutant defective for both enzymes (Δ*cydB*-Δ*cydD*-Δ(*cyoA*-*cyoB*)::*kan*). We also assayed Δ*cyxA*::*kan* and Δ*cyxB*::*kan* from the Keio collection to assess whether defective cytochrome *bd* oxidase II (Brondsted & Atlung 1996) affected larvae. For anaerobic respiration we generated a Δ*narZ*-Δ*narG*::*kan* double mutant to eliminate genes encoding subunits for both primary and secondary nitrate reductase, while periplasmic nitrate reductase was eliminated by generating a double mutant (Δ(*napA*-*napD*)::*kan*) lacking genes for the assembly protein and large reductase subunit (Stewart et al. 2002). Only larvae inoculated with the mutants defective for cytochrome *bd* oxidase (Δ*cydB*-

Δ*cydD*::*kan* or Δ*cydB*-Δ*cydD*-Δ(*cyoA*-*cyoB*)::*kan*) failed to molt (Figure 4.1D). Increasing the number of Δ*cydB*-Δ*cydD*::*kan* bacteria used to inoculate cultures did not alter this outcome while comparison to controls inoculated with wild-type *E. coli* showed no differences in the abundance of viable bacteria per larva (Figure 4.1E). These results identified the cytochrome *bd* oxidase respiratory pathway as essential for molting of *Ae. aegypti* first instars, and showed that the molt-defective phenotype for Δ*cydB*-Δ*cydD*::*kan E. coli* was not conditional over a range of inoculation densities.

Cytochrome *bd* oxidase dependent respiration cyclically reduces oxygenation of the larval gut

Since larvae inoculated with Δ*cydB*-Δ*cydD*::*kan E. coli* did not molt, we considered the possibility that aerobic respiration by bacteria alters oxygen availability in the gut. This was assessed by monitoring larvae for 48 h post-hatching using a hypoxia marker (Image-iT) that fluoresces with increasing intensity as atmospheric oxygen falls below 5%. Image iT fluorescence was detected at 6 h post hatching in the midgut of conventional and gnotobiotic larvae inoculated with wild-type *E. coli* (Figure 4.2; Figure C.1). Fluorescence intensity increased at 12 h but then declined after larvae achieved critical size (16 h) to undetectable levels at 24 h when molting to the second instar

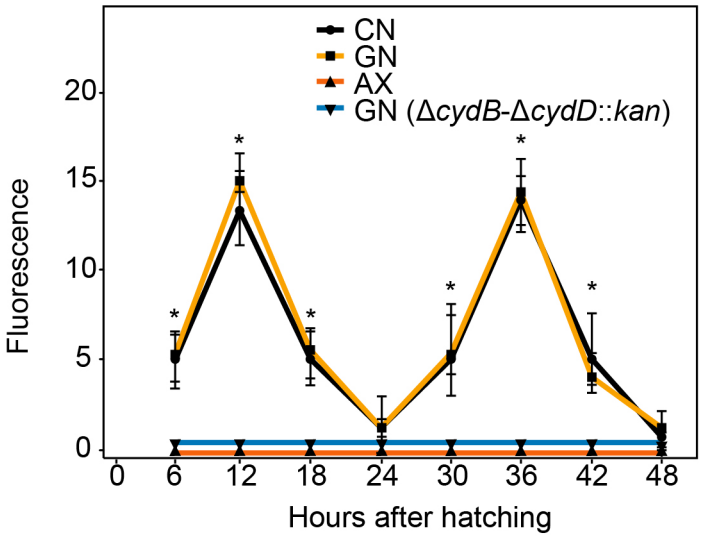


Figure 4.2. Quantitation of Image iT fluorescence in the midguts of conventional larvae (CN), gnotobiotic larvae inoculated with wild-type *Escherichia coli* (GN), axenic larvae (AX), and gnotobiotic larvae inoculated with Δ*cydB*-Δ*cydD*::*kan E. coli* (GN (Δ*cydB*-Δ*cydD*::*kan*)). Individual larvae for each treatment were examined by confocal microscopy from 6-48 h post-hatching. Fluorescence intensity in the midgut was measured in 10 larvae per treatment and time point using the Pixel Intensity plug-in from Image J software. Mean pixel intensity values ± SD are presented. * at each time point indicates that pixel intensity significantly differed for the CN and GN treatments relative to the AX and GN (Δ*cydB*-Δ*cydD*::*kan*) treatments (ANOVA followed by a post hoc Tukey-Kramer HSD test, $P < 0.05$). See Figure C.1 and C.2 for corresponding confocal images.

occurred (Figure 4.2; Figure C.1). Second instars exhibited the same pattern as larvae fed, achieved critical size at ~40 h, and molted to the third instar at 48 h (Figure 4.2; Figure C.1). In contrast, axenic larvae and gnotobiotic larvae inoculated with Δ*cydB*-Δ*cydD*::*kan E. coli* remained first instars and exhibited no gut hypoxia signal at any time point (Figure 4.2; Figure C.2). Prior results showed that the abundance and distribution of bacteria in the guts of conventional and wild-type *E. coli* gnotobiotic

larvae were indistinguishable with all bacterial cells in the midgut residing in the peritrophic space formed by the peritrophic matrix. Using an anti-peptidoglycan antibody to label bacteria and Hoechst 33342 to label gut cell nuclei, we observed that the distribution of Δ*cydB*-Δ*cydD*::*kan* bacteria in the guts of larvae at 12 h post-hatching was indistinguishable from conventional and wild-type gnotobiotic larvae (Figure C.3). Loss of cytochrome *bd* oxidase therefore had no effect on the abundance and distribution of *E. coli* in the midgut but it prevented oxygen levels from falling below 5%.

Given the absence of a hypoxia signal in larvae containing Δ*cydB*-Δ*cydD*::*kan E. coli*, we hypothesized that the rise in oxygen levels that occurs in post-critical size conventional and wild-type *E. coli* gnotobiotic larvae occurred because aerobic respiration by bacteria declined. To examine this, we first determined that the pH of the culture environment was near neutral (pH 6.9), whereas the pH of the anterior midgut was above 10 in both pre- (2 h post-hatching) and post-critical size (16 h) first instars (Figure 4.3A). This high midgut pH was consistent with earlier data showing that fourth instar *Ae. aegypti* maintain a gut lumen alkalization profile and exhibit an anterior midgut pH of 11 (Boudko et al. 2001; Corena et al. 2002). We then conducted assays that used black polystyrene microspheres in food and *E. coli* K-12 expressing

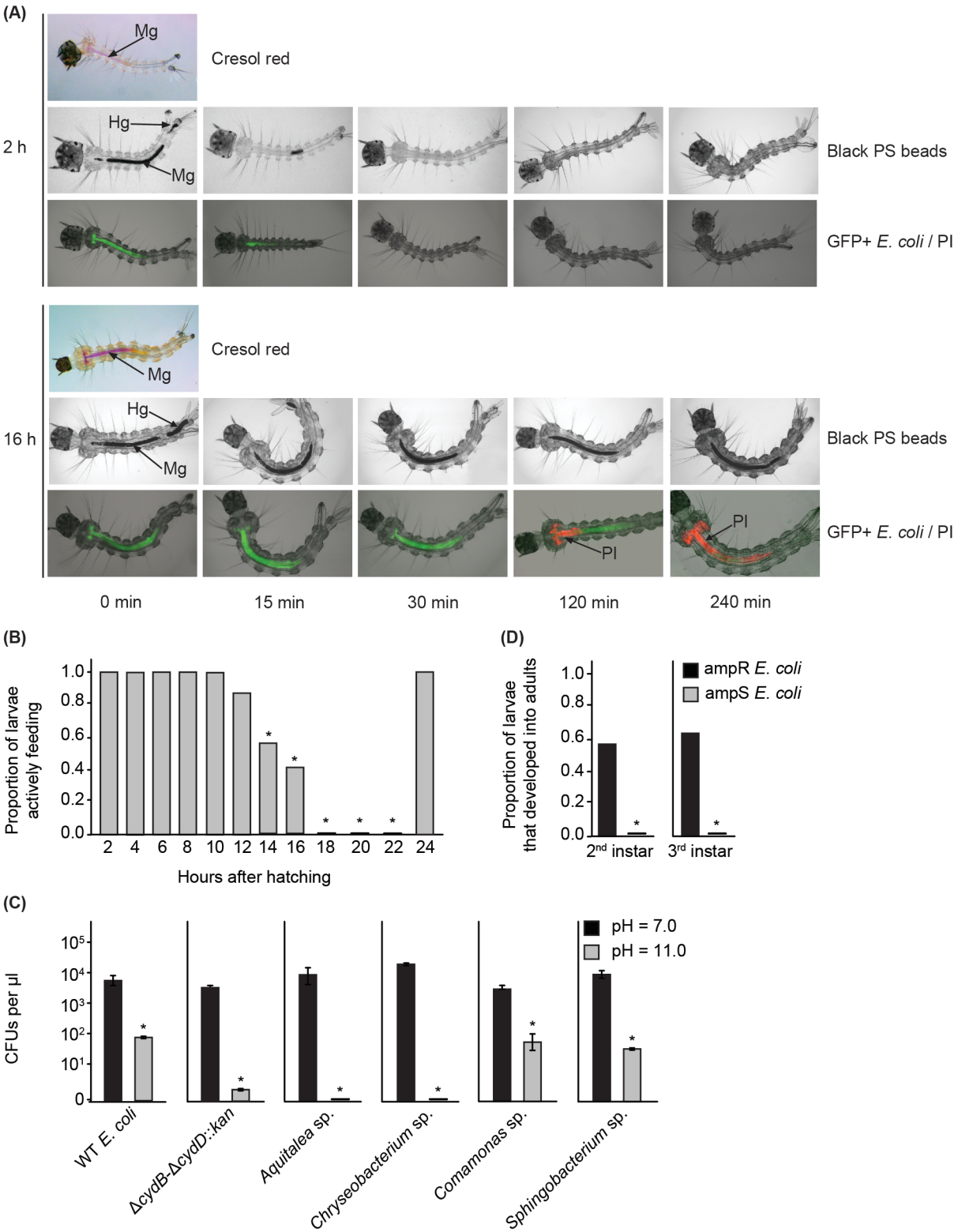

(A)
Mg
Cresol red
2 h
Hg
Mg
Black PS beads
GFP+ E. coli / PI
16 h
Pl
0 min
15 min
30 min
120 min
240 min
(B)
Proportion of larvae actively feeding
Hours after hatching
(D)
Proportion of larvae that developed into adults
ampR E. coli
ampS E. coli
2nd instar
3rd instar
(C)
CFUs per µl
pH = 7.0
pH = 11.0
WT E. coli
ΔcydB-ΔcydD::kan
Aquitalea sp.
Chryseobacterium sp.
Comamonas sp.
Sphingobacterium sp.

Figure 4.3. Excretion rates and viability of bacteria in the gut differ between pre- and post-critical size larvae. (A) Images showing midgut pH and excretion of ingested food and bacteria by pre- (2 h) and post-critical size (16 h) first instars. The uppermost panels at 2 and 16 h show first instars after ingestion of the pH indicator cresol red. The magenta color in the anterior midgut (Mg) indicates a strongly alkaline pH (> 10) which transitions to yellow and colorless in the posterior midgut (pH < 7.2). The middle panels show the movement of black polystyrene (PS) beads through the gut. The Mg and hindgut (Hg) contain beads at 0 min in 2 and 16 h larvae. All beads have been excreted by 30 min in 2 h larvae, while beads in the midgut remain present at 240 min in 16 h larvae. The lower panels show larvae that have ingested *Escherichia coli* expressing green fluorescent protein (GFP+) in cultures that also contain propidium iodide (PI). The Mg contains bacteria at 0 min in 2 and 16 h larvae. All bacteria have been excreted by 30 min in 2 h larvae with no staining by PI while bacteria are present but labeled with PI (orange) at 240 min in 16 h larvae. All larvae shown are ~200 μm in length. (B) Proportion of larvae from 2-24 h post-hatching that excreted all PS beads within 30 min. * indicates time points where the proportion of larvae that have excreted beads in 30 min is significantly lower than the 2 h time point ($P < 0.0001$, Fisher's exact test). At least 29 larvae were assayed per time point. (C) Sensitivity of wild-type (WT) *E. coli*, Δ*cydB*-Δ*cydD*::*kan E. coli*, and four abundant bacterial species present in conventionally reared larvae (*Aquitalea* sp., *Chryseobacterium* sp., *Comamonas* sp., and *Sphingobacterium* sp.) to culture at pH 7 versus pH 11. Bacteria were incubated for 2 h in neutral or alkaline LBK medium and then plated on neutral LB agar to determine the number of colony-forming units (CFUs) per μl. For each species, an * indicates a significant difference between treatments (t-test, $P < 0.01$). Three replicate cultures were tested per species and pH treatment. (D) Proportion of larvae that molted and successfully developed into adults when inoculated with ampicillin-susceptible (ampS) or ampicillin-resistant (ampR) WT *E. coli* and subsequently treated with ampicillin. Larvae were antibiotic treated immediately after molting to the 2nd or 3rd instar. * indicates a significant difference between treatments ($P < 0.0001$, Fisher's exact test). At least 60 larvae were assayed per instar and treatment.

green fluorescent protein (GFP+) to measure how rapidly larvae excreted consumed food and bacteria. At 2 h post-hatching larvae excreted food and bacteria within 30 min, whereas at 16 h ingested beads and bacteria persisted in the midgut (Figure 4.3A).

Using propidium iodide (PI) as a vital marker further showed no mortality of bacteria in 2 h post-hatching larvae, while viability of bacteria in 16 h larvae dramatically declined after 2 h (Figure 4.3A). The same assay conducted over the course of the first instar indicated that larvae excreted beads and bacteria within 30 min of ingestion from 2-10 h post-hatching, while post-critical size (16 h) larvae excreted little or no food and bacteria until molting (Figure 4.3B). Altogether, these data indicated that larvae continuously ingested and excreted food plus bacteria prior to achieving critical size, but ceased feeding and retained ingested food plus bacteria after achieving critical size. In turn, viable bacteria reduced midgut oxygen levels in pre-critical size larvae, but mortality of bacteria in post-critical size larvae caused oxygen levels to rise.

Most non-extremophilic bacteria grow over a range of external pH values (5.5-9.0) but exhibit rapid loss of viability under higher and lower pH conditions (Padan et al. 2005). This strongly suggested the decline in bacterial viability in post-critical size larvae occurred because of longer exposure to high midgut pH. We directly tested this by incubating four of the most abundant species of bacteria in conventionally reared *Ae. aegypti* (Coon et al. 2014) plus wild-type *E. coli* for 2 h in Luria Broth K (LBK) at pH 11. Results indicated the viability of each species was reduced 90% or more relative to bacteria cultured at pH 7 (Figure 4.3C). We also assessed whether *Ae. aegypti* required living bacteria for growth and molting after the first instar by inoculating axenic larvae

with wild-type *E. coli* that were susceptible or resistant to ampicillin. Larvae were then antibiotic treated immediately after molting to the second or third instar. Plate assays confirmed that ampicillin rapidly eliminated susceptible but not resistant bacteria from second and third instars (Figure C.4). In turn, no antibiotic-treated second and third instars inoculated with susceptible bacteria ever molted, while most antibiotic-treated larvae inoculated with resistant bacteria molted to the next instar and completed development into adults (Figure 4.3D). These findings were overall consistent with the idea that bacteria-mediated gut hypoxia functions as an essential signal in growth and molting of all instars.

An oral inhibitor of prolyl hydroxylases and 20-hydroxyecdysone stimulate molting

Cellular responses to hypoxia are primarily mediated by highly conserved α/β heterodimeric Hypoxia Inducible Transcription Factors (HIFs) (Semenza 2007; Dekanty et al. 2010). The HIFβ subunit is constitutive while specific prolyl hydroxylases (PHDs) hydroxylate HIFα under normoxia, which targets it for degradation (Semenza 2007; Dekanty et al. 2010). In contrast, PHD hydroxylation does not occur under hypoxia, which stabilizes HIFα/β to activate hypoxia-responsive genes (Semenza 2007). The small molecule FG-4592 is an oral inhibitor of HIF PHDs that stabilizes HIFα/β and triggers a

hypoxia transcriptional program under normoxic conditions (Rabinowitz 2013; Fluck & Faudry 2016; Jain et al. 2016). Transcriptional profiling indicated that HIFα, HIFβ, and PHD orthologs in *Ae. aegypti* were expressed in the gut and body wall of conventional, gnotobiotic larvae inoculated with wild-type *E. coli*, and axenic first instars (Vogel et al. 2017). We thus reasoned that FG-4592 would mimic the effects of wild-type bacteria if hypoxia signaled molting through a HIF transcriptional program. FG-4592 treatment significantly but only modestly increased the percentage of axenic larvae and gnotobiotic larvae inoculated with Δ*cydB*-Δ*cydD*-Δ(*cyoA*-*cyoB*)::*kan E. coli* that molted (Figure 4.4A). However, it greatly increased the percentage of gnotobiotic larvae inoculated with Δ*cydB*-Δ*cydD*::*kan E. coli* that molted to levels approaching wild-type *E. coli* (Figure 4.4A). We next added 20-hydroxyecdyone (20E) to cultures containing axenic or gnotobiotic larvae inoculated with Δ*cydB*-Δ*cydD*::*kan E. coli* on the premise that FG-4592 activated a hypoxia transcriptional program that results in release of ecdysone. Like FG-4592, 20E induced a much larger percentage of Δ*cydB*-Δ*cydD*::*kan E. coli* larvae to molt than axenic larvae (Figure 4.4B).

To investigate whether transient exposure to a hypoxic environment induced molting, we incubated axenic larvae for 2 h in 21% to less than 1% atmospheric oxygen. The percentage of axenic larvae that initiated a molt increased to 42% as oxygen levels

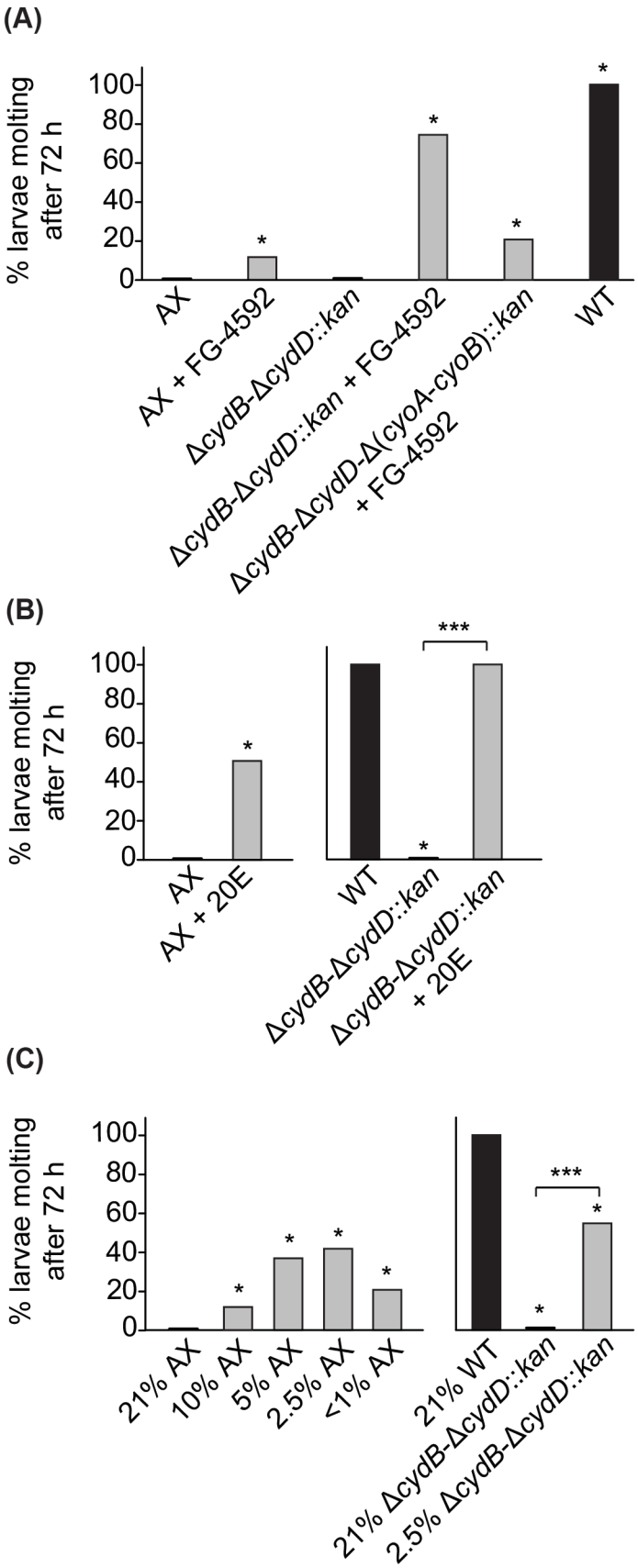

(A)
% larvae molting after 72 h
100
80
60
40
20
0
AX
AX + FG-4592
ΔcydB-ΔcydD::kan
ΔcydB-ΔcydD::kan + FG-4592
ΔcydB-ΔcydD-Δ(cyoA-cyoB)::kan + FG-4592
WT
(B)
% larvae molting after 72 h
***
AX
AX + 20E
WT
ΔcydB-ΔcydD::kan
ΔcydB-ΔcydD::kan + 20E
(C)
% larvae molting after 72 h
***
21% AX
10% AX
5% AX
2.5% AX
<1% AX
21% WT
21% ΔcydB-ΔcydD::kan
2.5% ΔcydB-ΔcydD::kan

Figure 4.4. Effects of FG-4592, 20E, and environmental hypoxia on axenic (AX) and gnotobiotic larvae inoculated with Δ*cydB*-Δ*cydD*::*kan* or Δ*cydB*-Δ*cydD*-Δ(*cyoA*-*cyoB*)::*kan Escherichia coli*. (A) Percentage of axenic (AX) and gnotobiotic larvae inoculated with Δ*cydB*-Δ*cydD*::*kan*, Δ*cydB*-Δ*cydD*-Δ(*cyoA*-c*yoB*)::*kan* or wild-type (WT) *E. coli* that molted to the second instar in the presence and absence of FG-4592. An * indicates a given treatment significantly differed from untreated AX larvae (negative control) ($P < 0.01$, Fisher's exact test). (B) Percentage of axenic (AX) (left panel) and Δ*cydB*-Δ*cydD*::*kan* gnotobiotic first instars (right panel) that molted to the second instar when treated with 20E. * in the left panel indicates the treatments significantly differed ($P < 0.01$, Fisher's exact test). * in the right panel indicates untreated Δ*cydB*-Δ*cydD*::*kan* larvae significantly differed from larvae inoculated with WT *E. coli* ($P < 0.01$, Fisher's exact test). (C) Percentage of axenic or gnotobiotic larvae inoculated with Δ*cydB*-Δ*cydD*::*kan E. coli* that molted to the second instar after transient exposure to environmental hypoxia. In the left panel, axenic larvae were maintained at 21% oxygen (normoxia) or exposed for 2 h to 10%-<1% oxygen. * indicates a significant difference in the proportion of larvae that initiated a molt relative to larvae maintained at 21% oxygen, which served as the negative control ($P < 0.0001$, Fisher's exact test). In the right panel, larvae inoculated with WT *E. coli* were maintained at 21% oxygen while larvae inoculated with Δ*cydB*-Δ*cydD*::*kan E. coli* were either maintained in 21% oxygen or exposed for 2 h to 2.5% oxygen. * indicates that Δ*cydB*-Δ*cydD*::*kan* larvae exposed to 21% and 2.5% oxygen differed from the WT positive control ($P < 0.0001$, Fisher's exact test). *** indicates that significantly more Δ*cydB*-Δ*cydD*::*kan* larvae exposed to 2.5% oxygen initiated a molt than Δ*cydB*-Δ*cydD*::*kan* larvae held in 21% oxygen. At least 40 larvae were assayed per treatment in (A), (B), and (C).

decreased to 2.5%, but this response declined to 21% at less than 1% oxygen (Figure 4.4C). Fifty five percent of gnotobiotic larvae inoculated with Δ*cydB*-Δ*cydD*::*kan E. coli* also initiated a molt at 2.5% oxygen (Figure 4.4C). Unlike treatment with FG-4592 or 20E, however, almost all larvae exposed to environmental hypoxia failed to ecdyse. Lastly, detritus feeding insects like mosquito larvae, herbivores, and other insects that consume carbohydrate-rich diets host bacteria that can produce fermentation products

including acetate, formate, lactate and butyrate (Engel & Moran 2013). These products have also been implicated in affecting developmental processes in both vertebrates and invertebrates (Engel & Moran 2013; Sommer & Backhed 2013). However, adding each to cultures of axenic or gnotobiotic first instars inoculated with Δ*cydB-cydD*::*kan E. coli* over a range of concentrations resulted in no larvae initiating or completing a molt.

4.5 Discussion

Insects known to require symbionts for survival live on nutrient poor diets where microbes provision essential resources and/or facilitate digestion (Engel & Moran 2013). They also involve obligate associations where particular species of microbes are directly transferred from parents to offspring or between individuals of the same insect species (Engel & Moran 2013, Wilson & Duncan 2015). Mosquito larvae can experience resource limitation in the field due to the habitats they occupy and/or competition (Rowbottom et al. 2015). However, they present the paradoxical situation of never developing under axenic conditions but naturally hosting variable communities of environmentally acquired microbes. We initially speculated that ingested microbes are simply digested and used as a source of nutrients. It was also plausible that multiple bacteria share traits that allow them to facilitate digestion or provide nutrients mosquitoes require. However, previous experiments strongly suggested these explanations are not why

conventional and gnotobiotic larvae develop very similarly when fed a nutritionally complete diet while axenic larvae die as first instars. We therefore hypothesized that microbes produce a cue in the gut that mosquitoes require to develop. Our genetic screen of *Escherichia coli* identified cytochrome *bd* oxidase as essential for growth and molting of *Aedes aegypti* larvae. Our functional data further indicate: (i) this enzyme is essential for reducing midgut oxygen levels below 5%, (ii) hypoxia functions as a developmental signal that is transduced through a hypoxia transcriptional program, and (iii) hypoxia-initiated signaling likely triggers ecdysone release through currently unknown pathway intermediates.

That multiple species of bacteria rescue development of *Ae. aegypti* is consistent with the widespread distribution of the cytochrome *bd* respiratory pathway including its presence in most gram-negative heterotrophs (Junemann 1997; Hao & Golding 2006). It is also possible aerobic respiration by non-bacterial members of the gut community such as fungi could contribute to producing a hypoxia signal in the larval midgut. While larvae inoculated with Δ*cydB*-Δ*cydD*::*kan E. coli* never molt, the presence of this mutant notably enhances molting induced by FG-4592 and 20E. Previous transcriptome data show that axenic larvae exhibit pronounced alterations in expression of genes with functions in nutrient acquisition and assimilation (Vogel et al. 2017). This suggests that bacteria including Δ*cydB*-Δ*cydD*::*kan E. coli* could promote nutrient assimilation

through unknown factors that function independently of microbe-induced hypoxia. Alternatively, low affinity cytochrome bo_3 oxidase activity in Δ*cydB*-Δc*ydD*::*kan E. coli* could produce an intermediate level of hypoxia in the gut that promotes nutrient acquisition and/or assimilation. Our results showing that FG-4592 induced a similarly weak molting response in gnotobiotic larvae inoculated with Δ*cydB*-Δ*cydD*-Δ(*cyoA*-*cyoB*)::*kan E. coli* as axenic larvae lends support for the latter suggestion.

The literature at-large distinguishes between microbes that establish residency in the digestive tract (autochthonous) versus microbes that are transiently present (allochthonous). In vertebrates, resident community members are usually more closely associated with the gut epithelium while transient community members are in the lumen with food (Nava & Stappenbeck 2011). The digestive tract of insect larvae including mosquitoes differs from vertebrates in two ways that affect the gut microbiota. First, the fore- and hindgut are lined by cuticle while epithelial cells secrete a peritrophic matrix that lines the midgut (Strand 2017). Together, these barriers prevent many microbes from directly contacting gut epithelia. Second, molting results in shedding and replacement of these barriers and is associated with shifts in feeding activity. All midgut bacteria in conventional and gnotobiotic *Ae. aegypti* larvae reside inside the peritrophic space (Vogel et al. 2017), while results of the current study show that a large proportion of these bacteria die after larvae achieve critical size. The consequence is that bacteria

induced midgut hypoxia rises in pre-critical size larvae but declines in post-critical size larvae due to pH-mediated death of bacteria. We also hypothesize these shifts in gut oxygen levels affect processes including insulin, TOR and 20E signaling, which have well-known functions in regulating insect growth and molting (Baker & Thummel 2007; Nijhout et al. 2013). Prior results show that mosquito larvae contain a subset of the microbial species in their environment (Boissiere et al. 2012; Coon et al. 2014; Gimonneau et al. 2014; Duguma et al. 2015; Muturi et al. 2016), but this study further refines this view by showing that most community members do not establish residency. Gut alkalization has also primarily been viewed as an adaptation by herbivores for digesting dietary protein and lowering binding tannins (Barbehenn & Constabel 2011). However, our data indicate that high midgut pH plays a critical role in phasic regulation of bacteria-mediated hypoxia.

We think it likely that gut hypoxia is an essential cue for growth and molting in all mosquitoes given prior results showing that axenic larvae from several species exhibit the same defects as *Ae. aegypti* (Coon et al. 2014; 2016a,b). We also speculate that microbe-mediated gut hypoxia could play an important regulatory role in other aquatic insects, because many taxa share similarities with mosquitoes in how they develop. In contrast, it is unclear whether gut microbes have such roles in terrestrial species. Oxygen supply has been implicated in regulating body size of terrestrial insects through

interaction with the tracheal system (Callier & Nijhout 2011). Unlike mosquitoes though, *Drosophila melanogaster* gut microbes are not essential for survival since axenic cultures can be maintained over multiple generations when fed a nutritionally complete diet (Bakula 1969). Only under conditions of low nutrient availability do axenic *Drosophila* larvae exhibit delays in development (Bakula 1969; Shin et al. 2011; Storelli et al. 2011; Ridley et al. 2012; Broderick et al. 2014). One study links restoration of normal development under nutrient poor conditions to acetate production by a gut community member (Shin et al. 2011), while another implicates a second community member in nutrient assimilation (Storelli et al. 2011). Our results do not support a role for acetate or other fermentation products in growth of *Ae. aegypti*, but as noted above our data do suggest a role for gut bacteria in nutrient acquisition and/or assimilation. In summary, our results identify bacteria-induced gut hypoxia as an essential cue for mosquito development, and provide a foundation for understanding how hypoxia signaling interfaces with the endocrine system to regulate growth and molting. Our results also identify a prominent group of insects that require gut microbes for survival but show no evidence that individual species and their gut microbiota form coevolved, integrated associations (holobiome) (Ziber-Rosenberg & Rosenberg 2008; Moran & Sloan 2015).

4.6 Materials and Methods

Mosquitoes and bacteria

UGAL *Aedes aegypti* larvae were reared on a nutritionally complete diet consisting of rat chow, lactalbumin, and inactive torula yeast (1:1:1) sterilized by gamma irradiation (Riehle & Brown 2002; Coon et al. 2014). Axenic larvae hatched from eggs that were surface sterilized as previously described (Coon et al. 2014). The K-12 single gene knockout library (Keio collection) (Baba et al. 2006) and select ASKA strains (Kitagawa et al. 2005) containing plasmids with genes of interest were obtained from the Nara Institute of Science and Technology (Nara, Japan). Double and quadruple gene deletions were constructed by either P1 phage transduction (Purdy et al. 2007) or the phage-λ red recombinase method (Datsenko & Wanner 2000) (Table C.2; Table C.3). Cells were grown in Luria Broth (LB) at 37 °C with 25 μg/ml kanamycin (Keio mutants) or 30 μg/ml chloramphenicol plus 50 μM isopropyl beta-D-thiogalactoside (IPTG) (ASKA clones).

Forward genetic screen and assays

Axenic larvae were individually placed in wells of 24-well culture plates (Corning) containing 1 ml of sterile water and 100 μg of standard mosquito rearing diet sterilized

by gamma irradiation (Coon et al. 2014). Each mutant from the Keio collection was grown to mid-log phase (OD_{600} ≈ 0.5-0.8) and inoculated at 10^6 colony-forming units (CFUs) per well. A minimum of 30 larvae was bioassayed per mutant. Mutants where fewer than 100% of larvae molted after 48 h were further assessed by collecting 100 μl of water from each well and homogenizing the larva after surface sterilization in phosphate buffered saline. Water and larval samples were then added to LB agar plates containing kanamycin followed by colony counts after 24 h at 37 °C. Plasmids were isolated from ASKA collection clones and transformed into Keio mutants that were subclassified as rescue defective. Transformed *Escherichia coli* were grown to mid-logarithmic phase (OD_{600} ≈ 0.5-0.8) with IPTG (50 μM) and then added to 24 culture wells containing axenic larvae followed by determination of the proportion of larvae that molted within 48 h.

Bioassays with double and quadruple knockout mutants were performed differently due to reduced growth rates in LB and mosquito cultures under conditions used for screening the Keio library. Methods were therefore used that standardized cell growth and viability to comparable levels between mutants and wild type (WT) *E. coli* K-12. In brief, each mutant was grown in LB to mid-log phase (OD_{600} ≈ 0.5-0.8) followed by suspension in M9 minimal medium at 10^3 CFUs/μl. Sterilized diet (~100 μg/ml) was then added to each suspension, followed by addition to wells containing axenic larvae.

To further ensure that viable cell counts per well remained consistent between treatments, larvae were rinsed and moved to new wells containing a freshly prepared suspension every 4 to 48 h.

Gut hypoxia and pH

Conventional, axenic, and gnotobiotic larvae inoculated with either WT or Δ*cydB*-Δ*cydD*::*kan E. coli* were incubated in water containing rearing diet plus 1 μM Image-iT Hypoxia Reagent (Life Technologies) at specific intervals post-hatching. Larvae were then moved to 4 °C for 10 min to slow movement followed by slide mounting and imaging using a Zeiss LSM 710 confocal microscope. Images were processed using Adobe Photoshop CS6. Gut pH in first instars was visualized by addition of 2.5% cresol red (w/v) to cultures for 30 min followed by image acquisition using a Leica MZFLIII stereomicroscope, Canon EOS digital SLR camera, and Leica Application Suite software. Images were then processed using Adobe Photoshop CS6.

Excretion, bacterial viability, and antibiotic clearance

Conventional first instars were fed 0.1% black polystyrene microspheres (Polysciences, Inc.) or 10^6 CFUs of *E. coli* K-12 expressing green fluorescent protein (GFP+) in 24-well plates containing standard diet at 2 h intervals for 45 min. Larvae were then rinsed

three times in water and moved to new wells containing water and diet only. The vital dye propidium iodide (1 μg/ml) was added to water containing larvae fed GFP+ *E. coli* to distinguish viable from inviable bacteria in the gut. Movement of black microspheres and GFP+ *E. coli* through the gut was then monitored at 15 min intervals for 4 h. Images were captured using either a Leica stereo- or epifluorescent microscope fitted with digital cameras followed by processing using Adobe Photoshop CS6. Plates containing axenic larvae were inoculated with 10^6 CFUs of either ampicillin-susceptible (ampS) or ampicillin-resistant (ampR) *E. coli* K-12 and maintained as described above. Larvae were then treated with 25 mg/ml ampicillin (Sigma) immediately after molting to the second and third instar. The proportion of larvae that developed into adults was then recorded while cohorts of larvae were also homogenized 24 h post-treatment and cultured on LB plates at 37 °C for 24 h to determine CFUs per individual.

Compound and environmental hypoxia assays

Axenic and gnotobiotic larvae inoculated with Δ*cydB*-Δ*cydD*::*kan* or Δ*cydB*-Δ*cydD*-Δ(*cyoA*-*cyoB*)::*kan E. coli* were reared as described above followed by addition of 1 μM FG-4592 (Selleckchem) or 100 μM 20E (Sigma) to the culture. Other larvae were treated by addition of 10 μM-1 mM sodium acetate (Sigma), sodium butyrate (Sigma), or sodium formate (Sigma), or 0.2-2% (v/v) lactic acid. Larvae were then monitored to

determine the proportion that molted to the second instar. Untreated larvae served as negative controls, while gnotobiotic larvae inoculated with WT *E. coli* K-12 served as a positive control. Environmental hypoxia assays were conducted by placing axenic or gnotobiotic larvae inoculated with Δ*cydB*-Δ*cydD*::*kan E. coli* in 30 ml sterile glass culture tubes containing 1 ml of M9 medium. Tubes were then fitted with a gas-tight rubber stopper and aluminum seal and made anaerobic by purging with oxygen-free nitrogen gas for 45 s to displace dissolved oxygen. Ambient air was then injected into anaerobic tubes using a sterile syringe to create atmospheric oxygen levels that ranged from 21% to <1%. After 2 h, larvae were individually moved to culture wells containing 1 ml of M9 medium and 100 μg sterilized diet. The proportion of larvae that initiated a molt was then determined with axenic and gnotobiotic larvae inoculated with WT *E. coli* in 21% oxygen serving as the negative and positive controls, respectively.

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CHAPTER 5

GUT BACTERIA DIFFERENTIALLY AFFECT EGG PRODUCTION IN THE ANAUTOGENOUS MOSQUITO *AEDES AEGYPTI* AND FACULTATIVELY AUTOGENOUS MOSQUITO *AEDES ATROPALPUS* (DIPTERA: CULICIDAE)[4]

[4]Coon, K.L., Brown, M.R. and M.R. Strand. 2016. *Parasites & Vectors* 9:375. Reprinted here with permission of the publisher.

5.1 Abstract

Background: *Aedes aegypti* and *Ae. atropalpus* are related mosquitoes that differ reproductively. *Aedes aegypti* must blood-feed to produce eggs (anautogenous) while *Ae. atropalpus* always produces a first clutch of eggs without blood feeding (facultatively autogenous). We recently characterized the gut microbiota of *Ae. aegypti* and *Ae. atropalpus* that were reared identically in the laboratory. Here, we assessed the effects of specific members of the gut microbiota in *Ae. aegypti* and *Ae. atropalpus* on female fitness including egg production.

Methods: Gnotobiotic *Ae. aegypti* and *Ae. atropalpus* larvae were colonized by specific members of the gut microbiota. Survival, development time, size and egg production for each treatment was then compared to axenic and conventionally reared larvae.

Results: Most species of bacteria we tested supported normal development and egg production by *Ae. aegypti* but only one betaproteobacterium, a *Comamonas*, supported development and egg production by *Ae. atropalpus* to equivalent levels as conventionally reared females. *Aedes atropalpus* females colonized by *Comamonas* contained similar stores of glycogen and protein as conventionally reared females, whereas females colonized by *Aquitalea* did not. Small differences in bacterial loads were detected between gnotobiotic and conventionally reared *Ae. aegypti* and *Ae. atropalpus*,

but this variation did not correlate with the beneficial effects of *Comamonas* in *Ae. atropalpus*.

Conclusions: Specific members of the gut microbiota more strongly affected survival, size and egg production by *Ae. atropalpus* than *Ae. aegypti*.

Keywords: microbiota, development, reproduction, oogenesis, clutch size

5.2 Background

Most mosquito species are anautogenous, which means that after mating with males, adult females must consume at least one blood meal from a vertebrate host for each clutch of eggs they produce and lay (Clements 1992). Repeated cycles of blood feeding and egg production also underlie why several mosquito species have evolved into vectors that transmit pathogens that cause several serious diseases in humans and other animals. In contrast, some mosquito species are autogenous and produce eggs without blood feeding (Clements 1992). Facultatively autogenous species emerge as adults and lay a first clutch of eggs without blood feeding but must blood-feed to produce additional clutches. Obligately autogenous species never blood-feed.

Regulation of egg formation is best understood in the anautogenous mosquito *Aedes aegypti*, which vectors several human pathogens including the viruses that cause yellow fever, dengue fever and Zika virus disease. In brief, the fat body of newly

emerged *Ae. aegypti* females becomes competent to produce yolk proteins through the activity of juvenile hormone (Zhu et al. 2003; Noriega 2004). However, oogenesis remains arrested until a blood meal is taken, which triggers the release of two neurohormones from the brain, insulin-like peptides (ILPs) and ovary ecdysteroidogenic hormone (OEH), that stimulate the ovaries to produce ecdysone (Brown et al. 2008; Vogel et al. 2015). Ecdysone, ILPs and other factors regulate the fat body to produce yolk proteins that are packaged into primary oocytes to produce mature eggs (Riehle & Brown 1999; Wu & Brown 2006; Brown et al. 2008; Gulia-Nuss et al. 2011; Vogel et al. 2015). Nutrients in the blood meal together with teneral reserves acquired during larval feeding provide the resources needed to produce yolk proteins (Attardo et al. 2005; Telang et al. 2006; Brandon et al. 2008).

The rockpool mosquito, *Aedes atropalpus*, is closely related to *Ae. aegypti* (Wilkerson et al. 2015) but is facultatively autogenous. *Aedes atropalpus* females produce similar numbers of eggs in the first gonadotropic cycle as *Ae. aegypti*, but fully rely on nutrient reserves acquired from feeding during the larval stage to do so (Chambers & Klowden 1994; Su & Mulla 1997; Telang et al. 2006; Gulia-Nuss et al. 2015). ILPs and OEH play a similar role in stimulating the ovaries in *Ae. atropalpus* to produce ecdysone and the fat body to synthesize yolk proteins as determined for *Ae. aegypti* (Gulia-Nuss et al. 2012; 2015). What differs is that *Ae. atropalpus* females

release these neurohormones shortly after emerging as adults rather than in response to blood feeding (Gulia-Nuss et al. 2012; 2015). The mechanism(s) that triggers blood meal-independent release of these hormones remains unclear, although data suggest it is linked to nutrient sensing and the availability of sufficient teneral reserves to support egg production (Telang et al. 2006; Gulia-Nuss et al. 2012; 2015).

Microorganisms in the digestive tract can also influence nutrient acquisition by animals including insects (Engel & Moran 2013). Mosquitoes host low diversity bacterial communities in their digestive tract that consist primarily of gram-negative aerobes and facultative anaerobes (Wang et al. 2011; Boissiere et al. 2012; Osei-Poku et al. 2012; Coon et al. 2014; Gimonneau et al. 2014; Minard et al. 2014; Duguma et al. 2015; Muturi et al. 2016). Mosquitoes acquire most if not all of these bacteria from the aquatic habitat they develop in as larvae while transmitting some but not all community members to adults (Boissiere et al. 2012; Coon et al. 2014).

We recently used 16S rDNA sequencing to characterize the gut microbiota of *Ae. aegypti* and *Ae. atropalpus* larvae reared under identical environmental conditions in the laboratory. Results showed that similar communities of bacteria were present in the aquatic habitat of both species during larval development. However, Bacteroidetes (Flavobacteriaceae) and Actinobacteria (Microbacteriaceae) dominated the gut community in *Ae. aegypti* larvae, whereas Proteobacteria (Class Betaproteobacteria)

dominated the community in *Ae. atropalpus* (Coon et al. 2014). We developed methods to produce axenic (i.e. bacteria-free) larvae, which showed that both species fail to develop beyond the first instar in the absence of gut bacteria, but develop normally into adults if recolonized by bacteria in their laboratory aquatic habitat. We also developed methods to produce gnotobiotic larvae that were colonized by a particular species of bacterium. Bioassays showed that several species of bacteria present in the laboratory aquatic habitat could individually colonize axenic *Ae. aegypti* larvae, and that the resulting gnotobiotic larvae also develop normally into adults (Coon et al. 2014). However, no studies have examined the effects of the gut microbiota in larvae on egg production by *Ae. aegypti* or any fitness traits in *Ae. atropalpus*.

In this study, we produced gnotobiotic *Ae. aegypti* and *Ae. atropalpus* larvae that were singly colonized by abundant members of the larval gut microbiota and compared their effects on female-related fitness traits including egg production in the first ovarian cycle. Our results showed that gnotobiotic *Ae. aegypti* singly colonized by several members of the gut microbiota developed and produced eggs similarly to conventionally reared *Ae. aegypti* females with a mixed community of bacteria. In contrast, only one community member we tested rescued development and egg production to equivalent levels as conventionally reared *Ae. atropalpus* females.

5.3 Methods

Conventional rearing of *Aedes aegypti* and *Aedes atropalpus*

The UGAL strain of *Ae. aegypti* and Bass Rock strain of *Ae. atropalpus* were conventionally reared in the same insectary at 27 °C, 60% relative humidity, and 16 h light:8 h dark photoperiod (Gulia-Nuss et al. 2012). Larvae were fed a standard diet consisting of finely ground rat chow (Purina): lactalbumin: brewers yeast (1:1:1) in open aluminum rearing pans containing distilled water (Riehle & Brown 2002). Pupae were transferred from larval rearing pans to plastic cages for adult emergence. These methods produced large cohorts of adult *Ae. aegypti* and *Ae. atropalpus* that were similar in size. Adults were fed 10% sucrose in water (wt/vol) ad libitum. Adult female *Ae. aegypti* were blood fed two days post-emergence on an anesthetized rat until engorged. *Aedes aegypti* females laid a clutch of eggs approximately 36 h after blood feeding on damp filter paper. *Aedes atropalpus* females in contrast laid clutches of eggs on damp filter paper 3–5 days post-emergence.

Isolation and taxonomic assignment of bacteria from larvae

Bacterial isolates were maintained as summarized in Table D.1. Bacteria were isolated from conventionally reared *Ae. aegypti* and *Ae. atropalpus* larvae by collecting and

surface sterilizing fourth instars followed by homogenization in 1.6 ml centrifuge tubes in sterile phosphate buffered saline (PBS) using a plastic pestle (Coon et al. 2014). Homogenates were then plated on Luria Broth (LB), brain-heart infusion (BHI), tryptic soy agar (TSA), Reasoner's 2A (R2A), or blood agar plates at 27 °C for 24–72 h (Table D.1). Single colonies were picked for serial dilution streaking followed by DNA isolation from a single colony using the Gentra Puregene Yeast/Bact kit (Qiagen). After PCR amplification using universal primers (1492F and 129R in Table D.2) and cloning into a vector (pCR 2.1 TOPO TA cloning, Invitrogen), ~1,000 bp of the 16S–23S internal transcribed spacer (ITS) region was Sanger sequenced. Resulting sequence data were then used to design genus-specific primers for each isolate of interest (Table D.2). Specificity of primers for a given isolate was verified by PCR using DNA from each of the identified bacterial isolates as previously described (Coon et al. 2014).

Inoculation of axenic first instars with particular gut community members

Axenic larvae were produced by surface sterilizing eggs from the conventionally reared *Ae. aegypti* and *Ae. atropalpus* colonies followed by hatching of first instars in sterile water per Coon et al. (2014). Larval diet was sterilized by exposure to 5 kGy from a cobalt 60 gamma radiation source, while 10% sucrose was filtered sterilized for feeding

adults. First instars were inoculated with a particular species of bacterium by placing 20 axenic larvae in a 150×15 mm Petri dish containing water sterilized by autoclaving followed by addition of sterile diet and approximately 10^8 cells of a given bacterial isolate suspended in sterile water. Sterilized diet was thereafter added to dishes every other day until death or pupation. Pupae were surface-sterilized by placing in 2% (vol/vol) bleach for 2 min and rinsing 3 times in sterile water. Pupae were then placed in sterile water in an autoclaved polypropylene plastic chamber (Olcott Plastics) for adult emergence. The axenic status of larvae and food were confirmed by culture-based methods and PCR analysis using universal 16S rRNA primers (27F and 1492R in Table D.2) (Coon et al. 2014). The same methods were also used to determine the presence of bacteria or a particular isolate in water, larvae or adults.

Larval survival and development time assays

Dishes containing 20 first instars inoculated with a particular bacterial isolate were produced as described above. Dishes containing 20 axenic first instars with sterile food but no bacteria served as a negative control, while 20 conventional first instars in non-sterile water but fed sterile food served as the positive control. Dishes were then maintained under the same environmental conditions. The number of larvae that pupated per dish for each treatment was recorded daily. All pupae from a dish were

then removed, surface sterilized, and placed in sterile water in sterile containers as described above. Dead larvae and pupae were removed and discarded. Survival to adulthood was measured as the proportion of first instars that successfully emerged as adults. Between 5 and 30 replicate dishes were monitored for each treatment. With 20 larvae per replicate, we overall monitored from 100 to 600 first instars per treatment.

Adult size and mature egg production assays

Gnotobiotic and conventionally reared adult females were transferred to polypropylene cages (Olcott Plastics) lined with moist filter paper and containing a cotton wick soaked with water. Filter paper, wicks, water and cages were autoclaved before use. The number of eggs laid by each female was counted by visually inspecting the filter paper using a dissecting microscope. Each female was then dissected in PBS and the number of mature eggs remaining in the ovaries was also determined by visual inspection using previously established criteria (Gulia-Nuss et al. 2015). These criteria included that unlaid mature eggs had to be ≥ 300 μm in length and have a fully formed chorion. Egg production was quantified for individual, mated *Ae. atropalpus* females at 120 h post-emergence. Egg production was quantified for individual, mated *Ae. aegypti* females 72 h after blood feeding on a surface-sterilized rat. First clutch size of each female was defined as the total number of oviposited plus mature eggs remaining in the ovaries. The

size of each ovipositing female was then determined by measuring the length of the forewing from the axillary incision to the tip excluding fringe using an ocular micrometer. A minimum of 20 females per treatment was assayed.

Nutrient reserves in conventional and gnotobiotic *Aedes atropalpus*

Conventionally reared and gnotobiotic adult females were collected 12 h post-emergence followed by dissection in PBS and removal of the digestive tract. The remaining body wall, which contains the fat body, from two individuals was transferred to a microfuge tube and homogenized in 100 µl of water (protein assay) or 100 µl of Na_2SO_4 with 200 µl of methanol (glycogen, lipid assays) followed by storage at −80 °C before use (Zhou et al. 2004). After centrifugation (12,000 x *g*, 4 °C), supernatants were used to determine total protein, glycogen and lipid amounts as previously described (Brown et al. 2008; Gulia-Nuss et al. 2012). A total of 10 body wall pairs were assayed for each treatment.

Bacterial load in conventional and gnotobiotic *Aedes aegypti* and *Aedes atropalpus*

The number of bacteria in gnotobiotic larvae and adults was assessed by standard plate counts. Individual larvae were collected after molting to the fourth instar from replicate

dishes, surface-sterilized, homogenized in 100 µl of PBS as described above, serially diluted and plated on LB agar. Colony-forming units (CFUs) were then determined after incubation of plates at 27 °C for 24 h. Homogenates were also plated from adult females collected 6–12 h and either 24 h post-blood meal (*Ae. aegypti*) or 72 h post-emergence (*Ae. atropalpus*). At least 4 larvae or adults were analyzed per treatment with each sample internally replicated 4 times.

Statistical analysis

All analyses were performed using R (http://www.r-project.org/). Development time and total nutrient amounts were tested for normality and equality of variances before analysis by one-way analysis of variance (ANOVA), followed by post hoc comparisons using Dunnett's tests. Survival data were analyzed by Chi-square or Fisher's exact tests followed by post hoc Bonferroni-corrected pairwise tests to compare treatments. Bacterial counts were analyzed by ANOVA followed by either post hoc comparison to the conventional positive control using Dunnett's test or Tukey-Kramer Honest Significant Difference (HSD) tests. The number of mature eggs females produced was analyzed by Fisher's exact test to compare the proportion of females that produced one or more mature eggs in each bacterial treatment while the effects of size were analyzed

by logistic regression. These data were also examined by an analysis of covariance (ANCOVA) followed by post hoc comparisons using a Dunnett's test.

5.4 Results

Individual bacteria differentially affect *Aedes aegypti* and *Aedes atropalpus* development

We first compared development time to pupation and survival to adulthood of gnotobiotic larvae colonized by individual bacterial isolates to that of conventionally reared control larvae. We focused our assays on representative genera from each of the major bacterial phyla previously identified from the digestive tracts of our laboratory mosquitoes (Coon et al. 2014; Table D.1): *Paenibacillus* (Firmicutes: Paenibacillaceae), *Chryseobacterium* (Bacteroidetes: Flavobacteriaceae), *Sphingobacterium* (Bacteroidetes: Sphingobacteriaceae), *Microbacterium* (Actinobacteria: Microbacteriaceae), *Leucobacter* (Actinobacteria: Microbacteriaceae), *Aquitalea* (Proteobacteria: Neisseriaceae) and *Comamonas* (Proteobacteria: Comamonadaceae). Two of these genera, *Aquitalea* and *Comamonas*, belong to the Betaproteobacteria and were previously identified as dominant members of the *Ae. atropalpus* larval gut community by pyrosequencing. All other genera were previously isolated from *Ae. aegypti* (Coon et al. 2014; Table D.1).

Members of the Bacteroidetes (Flavobacteriaceae, Sphingobacteriaceae) and Actinobacteria (Microbacteriaceae) are commonly found in anautogenous species both in the lab and field (Wang et al. 2011; Boissiere et al. 2012; Osei-Poku et al. 2012; Coon et al. 2014; Gimonneau et al. 2014; Minard et al. 2014; Duguma et al. 2015; Muturi et al. 2016), and make up as much as 90% of the gut bacterial community in our laboratory mosquito cultures (Coon et al. 2014).

We collected eggs from conventionally reared *Ae. aegypti* and *Ae. atropalpus* and produced axenic (i.e. bacteria-free) first instars, which were either maintained in sterile water with sterile food or placed into water inoculated with one of the above isolates plus food. PCR screening of bacterial 16S rDNA using universal primers 6 h after hatching confirmed the presence of bacteria in conventionally reared first instars and the absence of bacteria in axenic larvae (Figure C.1). Screening with genus specific primers also confirmed that inoculation of axenic larvae with the above isolates resulted in larvae that contained each bacterium at 6 h post-inoculation (Figure C.1).

Results showed that 87% of *Ae. aegypti* and 82% of *Ae. atropalpus* larvae reared conventionally (= non-sterile) developed and emerged as adults at 8 days post-egg hatching (positive control), while as previously reported no axenic *Ae. aegypti* or *Ae. atropalpus* larvae developed beyond the first instar (negative control) (Figure 5.1A). All axenic *Ae. aegypti* and *Ae. atropalpus* that were inoculated with *Microbacterium* or

Leucobacter also died 4–5 days post-inoculation as first instars (Figure 5.1A). All of the other isolates tested supported survival to adulthood and development times for *Ae. aegypti* larvae that did not significantly differ from conventionally reared larvae (Figure 5.1A,B). In contrast, only *Aquitalea* and *Comamonas* supported survival and development times for *Ae. atropalpus* larvae that did not differ from conventionally reared *Ae. atropalpus* (Figure 5.1A,B). Survival of *Ae. atropalpus* larvae inoculated with *Paenibacillus*, *Chryseobacterium* or *Sphingobacterium* was intermediate between the negative and positive controls with development times for survivors also being longer than for conventionally reared *Ae. atropalpus* (Figure 5.1A,B). The reduced survival of *Ae. atropalpus* inoculated with *Paenibacillus*, *Chryseobacterium* or *Sphingobacterium* was due to a larger proportion of individuals dying as larvae ($\chi^2 = 18.9054$, $\mathrm{df} = 1$, $\mathrm{P} < 0.0001$) rather than a smaller proportion of pupae failing to emerge as adults ($\chi^2 = 0.1822$, $\mathrm{df} = 1$, $\mathrm{P} > 0.05$).

For the individuals in each treatment that developed into adults, we estimated female size by measuring forewing length, which has been used in several other studies of mosquitoes including *Ae. aegypti* and *Ae. atropalpus* to estimate body size (Van Handel & Day 1989; Nasci 1990; Telang et al. 2006). There were no differences in size between conventionally reared *Ae. aegypti* adult females and adults that emerged from larvae colonized by *Paenibacillus*, *Chryseobacterium*, *Sphingobacterium*, *Aquitalea* or

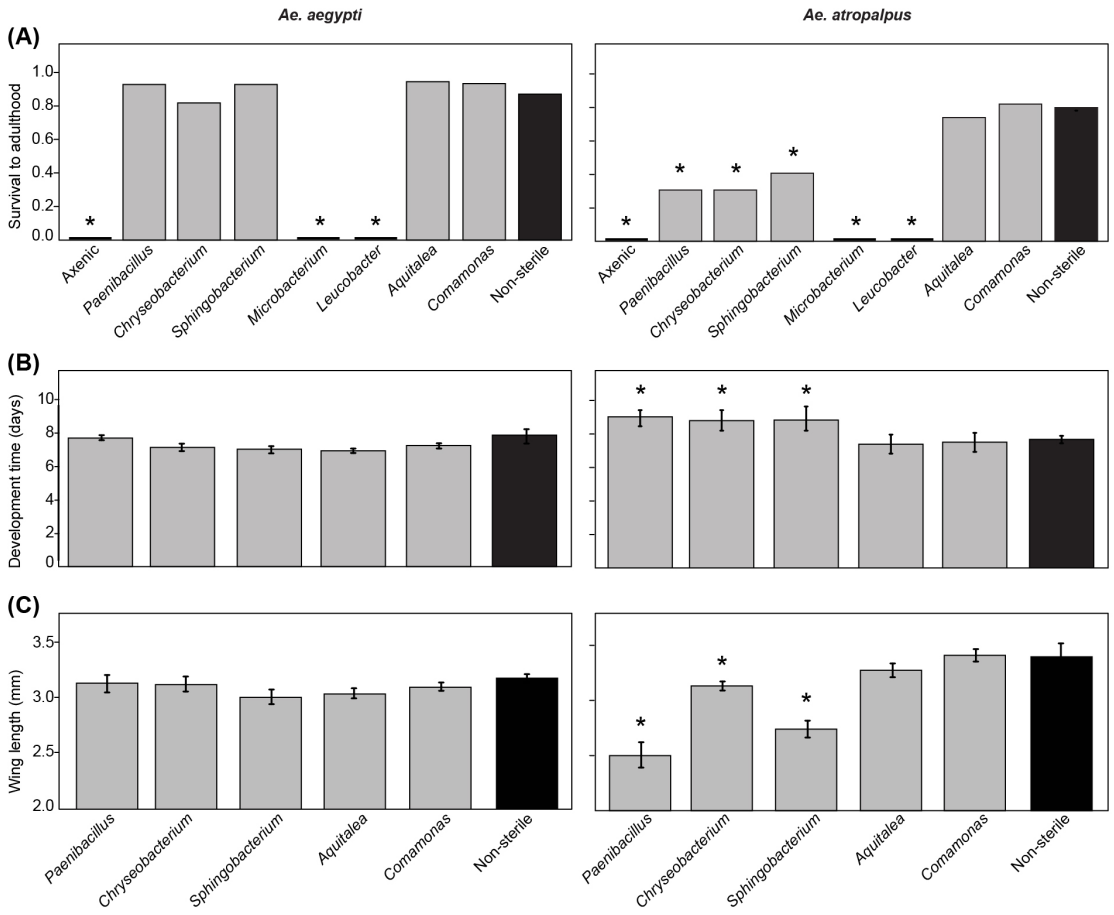


Figure 5.1. Development of *Aedes aegypti* and *Ae. atropalpus* larvae that were axenic, inoculated with a single bacterial species, or conventionally reared (non-sterile). (A) Survival from egg hatching to adult emergence differed among treatments for *Ae. aegypti* (Fisher's exact test: $P = 0.0005$) and *Ae. atropalpus* (Fisher's exact test: $P < 0.0001$). An asterisk (*) above a given bar indicates the treatment significantly differed from the non-sterile control by post hoc pair-wise comparisons with Bonferroni correction. (B) Development time from egg hatching to pupation differed among treatments for *Ae. aegypti* (ANOVA: $F_{(5,673)} = 8.0$, $P < 0.0001$) and *Ae. atropalpus* (ANOVA: $F_{(5,880)} = 211.3$, $P < 0.0001$). (C) Size as estimated by forewing length did not differ among treatments for *Ae. aegypti* (ANOVA: $F_{(5,183)} = 1.2$, $P = 0.29$) but did differ for *Ae. atropalpus* (ANOVA: $F_{(5,129)} = 32.7$, $P < 0.0001$). Asterisks above the bars in (B) or (C) indicate means that significantly differed from the non-sterile control as determined by Dunnett's test ($P < 0.01$). A minimum of 5 replicate dishes and 100 larvae per treatment were assayed for survival and development times. A single forewing from a minimum of 20 randomly selected adult females per treatment was measured to estimate adult size. The bars in (B) and (C) present mean values with 95% confidence intervals for each treatment.

Comamonas (Figure 5.1C). However, only gnotobiotic *Ae. atropalpus* larvae inoculated with *Aquitalea* or *Comamonas* developed into adults that did not significantly differ in size from conventionally reared *Ae. atropalpus* (Figure 5.1C). *Aedes atropalpus* adults produced from larvae inoculated with *Paenibacillus*, *Chryseobacterium* or *Sphingobacterium* were significantly smaller (Figure 5.1C).

Several bacteria support normal egg production in *Aedes aegypti* but not *Aedes atropalpus*

We next focused on those species of bacteria that supported development of at least some *Ae. aegypti* and *Ae. atropalpus* to adulthood by assessing their effects on egg production by females. For each treatment, females were mated with males from the same cohort and subsequently housed individually in sterile containers for 36–120 h. *Aedes aegypti* females were blood fed 2 days post emergence. Egg production data were recorded in three ways: (i) the proportion of females for each treatment that laid or contained at least one mature egg; (ii) the total number of mature eggs per female (laid plus present in the ovaries); and (iii) the total number of laid eggs.

For *Ae. aegypti*, all females in each treatment produced mature eggs (Figure 5.2A). All gnotobiotic females also produced the same total number of mature eggs ($F_{(5,183)} = 2.26,\ P > 0.05$) and laid the same number of eggs ($F_{(5,183)} = 2.19,\ P > 0.05$) as

conventionally reared females (Figure 5.2B,C). In contrast, there was a very strong treatment effect on the proportion of *Ae. atropalpus* females that produced any mature eggs (Fisher's exact test; $P < 0.0001$) (Figure 5.2A). Almost all conventionally reared females and females colonized by *Comamonas* or *Aquitalea* produced mature eggs, whereas $< 60\%$ of gnotobiotic females colonized by *Chryseobacterium* or *Sphingobacterium* produced eggs while only 23% of females colonized by *Paenibacillus* produced eggs (Figure 5.2A). We considered that these differences could reflect the effect of adult size rather than bacterium. However, regression analysis across all individuals showed that wing length did not strongly predict whether a female produced mature eggs ($P = 0.08$). For the *Ae. atropalpus* females in each treatment that produced mature eggs, we compared clutch sizes using an ANCOVA where wing length served as the covariate. We detected no significant interaction between wing length and treatment ($F_{(1,5)} = 0.9,\ P = 0.49$), indicating equivalent regression slopes for each treatment, while comparison of the adjusted treatment means indicated that clutch sizes significantly differed among treatments ($F_{(5,127)} = 13.0,\ P < 0.0001$). The total number of mature eggs produced by gnotobiotic females colonized by *Comamonas* did not significantly differ from the number of mature eggs produced by conventional females, whereas females colonized by *Aquitalea*, *Sphingobacterium*, *Chryseobacterium* and *Paenibacillus* all produced fewer mature eggs (Figure 5.2B). Across all treatments, *Ae. atropalpus* females

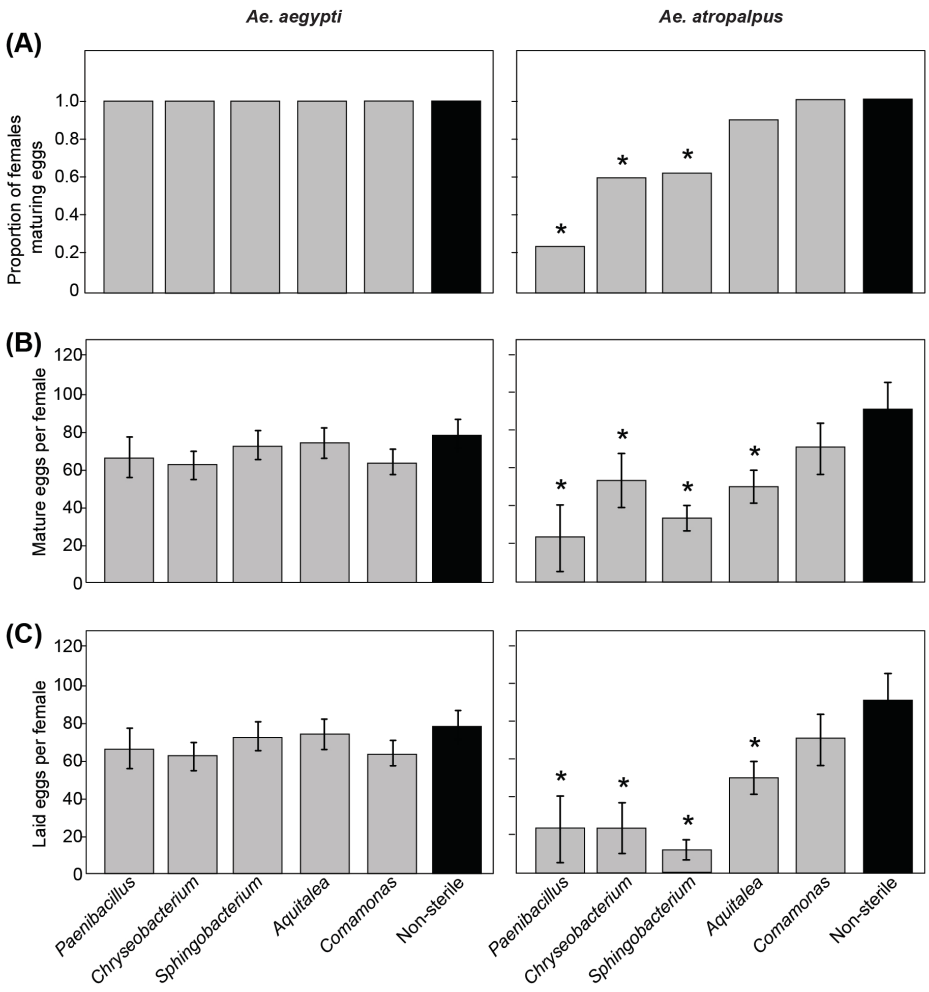


Figure 5.2. Mature egg formation by *Aedes aegypti* and *Ae. atropalpus* adult females from larvae that were inoculated with a single bacterial species or conventionally reared (Non-sterile). (A) The proportion of females that produced one or more mature eggs did not differ among treatments for *Ae. aegypti* (Fisher's exact test: $P > 0.05$) but did differ for *Ae. atropalpus* (Fisher's exact test: $P < 0.0001$). An asterisk above a given bar (*) indicates the treatment significantly differed from the non-sterile control by post hoc pair-wise comparisons with Bonferroni correction. (B) Total clutch sizes (sum of the number of eggs laid and the number of mature eggs in the ovaries) did not differ among treatments for *Ae. aegypti* (ANOVA: $F_{(5,183)} = 2.3$, $P > 0.05$) but did differ for *Ae. atropalpus* (ANOVA: $F_{(5,127)} = 13.0$, $P < 0.0001$). (C) Number of eggs laid by females in a given treatment that produced at least one mature egg did not not differ for *Ae. aegypti* (ANOVA: $F_{(5,183)} = 2.2$, $P > 0.05$) but did differ for *Ae. atropalpus* (ANOVA: $F_{(5,127)} = 18.4$, $P < 0.0001$). Bars in (B) and (C) present mean values with 95% confidence intervals while asterisks (*) in (B) and (C) indicate treatments that significantly differed from the non-sterile control (Dunnett's test; $P < 0.01$).

laid a majority of the mature eggs they produced (Figure 5.2C). For both *Ae. aegypti* and *Ae. atropalpus*, hatch rates of eggs laid by gnotobiotic females exceeded 90% and did not differ from eggs laid by conventionally reared females. Altogether then, treatment had no effect on egg production by *Ae. aegypti*, while in *Ae. atropalpus* the main difference between treatments was in the number of mature eggs females produced and laid rather than the viability of laid eggs.

Previous studies have measured the nutrient reserves (lipid, glycogen, protein) in conventionally reared *Ae. aegypti* and *Ae. atropalpus* females at adult emergence (Telang et al. 2006; Gulia-Nuss et al. 2015). We did not measure nutrient levels again in *Ae. aegypti* for this study because no differences in egg production were detected between gnotobiotic and conventionally reared females. However, we did compare nutrient reserves between gnotobiotic *Ae. atropalpus* colonized by *Aquitalea* or *Comamonas* and conventionally reared females. We also focused on these treatments because survival and adult female sizes were similar but egg production was lower in *Aquitalea*-colonized females. Results showed that stored lipid was higher in newly emerged conventional females than gnotobiotic females colonized by *Comamonas*, but protein and glycogen were equivalent (Figure 5.3). In contrast, lipid, protein and glycogen levels were all higher in conventional than gnotobiotic females colonized by *Aquitalea* (Figure 5.3).

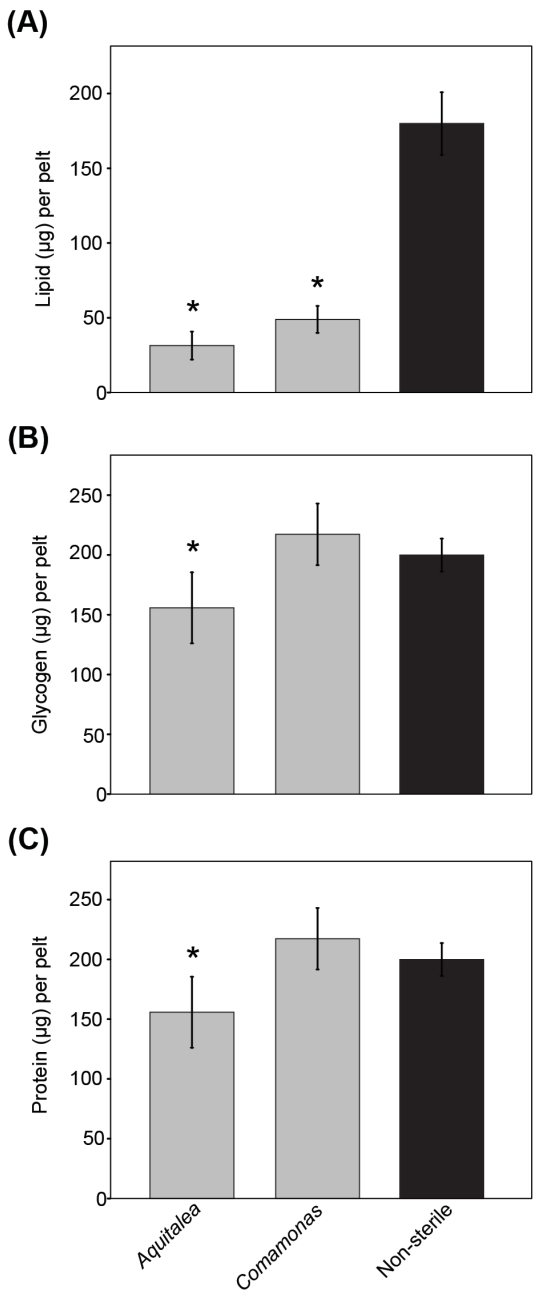


Figure 5.3. Total lipid (A), glycogen (B) and protein (C) in *Aedes atropalpus* adult females from larvae that were inoculated with *Aquitalea*, *Comamonas*, or conventionally reared (non-sterile). Lipid (ANOVA: $F_{(3,35)} = 96.0$, $P < 0.0001$), glycogen (ANOVA: $F_{(3,35)} = 6.2$, $P = 0.002$), and protein (ANOVA: $F_{(3,35)} = 6.2$, $P = 0.002$) significantly differed among treatments. For each nutrient, an asterisk above a bar indicates means significantly differed from the non-sterile control (Dunnett's test; $P < 0.01$). Two adult females with gut removed were analyzed per replicate with 10 replicates analyzed for each treatment and nutrient. Bars indicate mean values with 95% confidence intervals.

Bacterial loads in *Aedes aegypti* and *Aedes atropalpus* are similar

One possible explanation for the effects different gut community members have in gnotobiotic *Ae. aegypti* versus *Ae. atropalpus* is that bacterial abundance differed. We therefore assessed this by plating homogenates of larvae or adults after surface sterilization, which yielded CFUs per individual. Since larvae inoculated with *Microbacterium* or *Leucobacter* all died as first instars, we assayed first instars for these

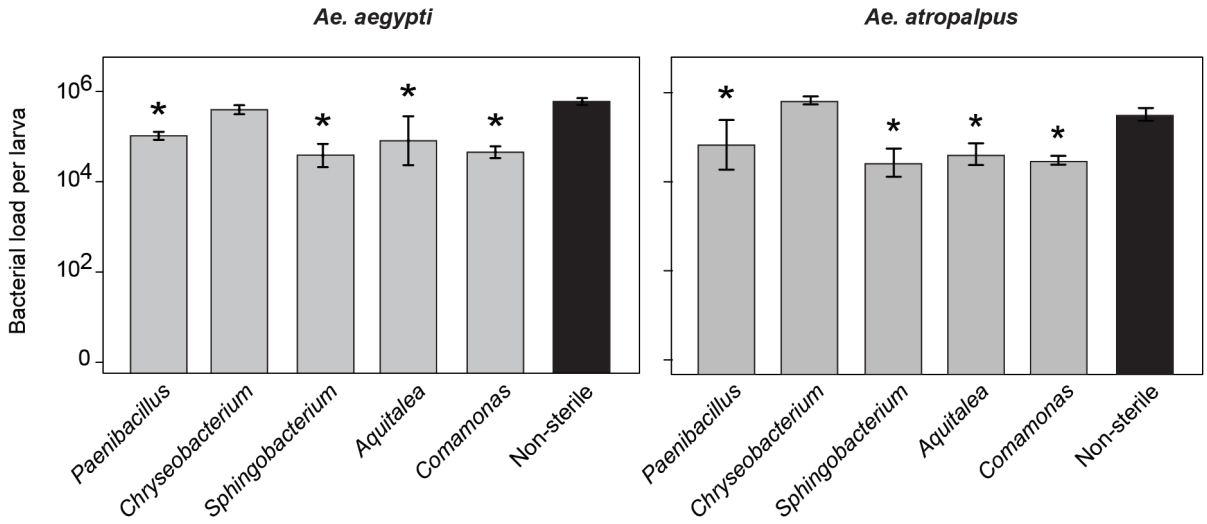


Figure 5.4. Bacterial loads in fourth instar gnotobiotic larvae colonized by a single bacterium and conventionally reared (non-sterile) fourth instars as measured by plate counts. Each gnotobiotic treatment and the conventionally reared (Non-sterile) control are indicated on the x-axis. A minimum of 4 individuals was assayed per treatment. Bars indicate mean bacteria per larva with 95% confidence intervals. Bacterial loads overall significantly differed among treatments for *Aedes aegypti* (ANOVA: $F_{(5,18)} = 13.9$, $P < 0.0001$) and *Ae. atropalpus* (ANOVA: $F_{(5,34)} = 12.4$, $P < 0.0001$). Asterisks (*) indicate treatments that significantly differed from conventionally reared larvae as determined by a post hoc Dunnett's test ($P < 0.01$).

treatments at three days post-inoculation. Plating assays yielded no colonies from any larva, which in light of the data in Figure D.1 indicated that *Ae. aegypti* and *Ae. atropalpus* first instars ingested these species but they failed to colonize. Since inoculation with the other isolates resulted in most larvae molting but varying numbers of *Ae. atropalpus* developing into adults, we first compared bacterial load in larvae that had just molted to the final (fourth) instar (0–6 h). PCR assays as in Figure D.1 showed that gnotobiotic *Ae. aegypti* and *Ae. atropalpus* fourth instars both contained the bacterium they were inoculated with. Colony counts indicated that each gnotobiotic treatment except *Chryseobacterium* contained fewer viable bacteria per larva than the conventionally reared (non-sterile) positive control for both mosquito species (Figure 5.4).

We then compared these larval data to bacterial loads in newly emerged (6–12 h) and 72 h old adults. For *Ae. aegypti*, 72 h old females had also blood fed 24 h previously. Like the larval samples, each gnotobiotic treatment plus the non-sterile control was measured in at least four individuals for the two adult sample times. For 6–12 h old and 72 h old adults of both species, colony counts for each gnotobiotic treatment did not differ from the non-sterile control except for *Chryseobacterium,* which was more abundant (Figure 5.5). Merging the data from each treatment and comparing between stages for each species indicated that bacterial loads in *Ae. aegypti* were highest

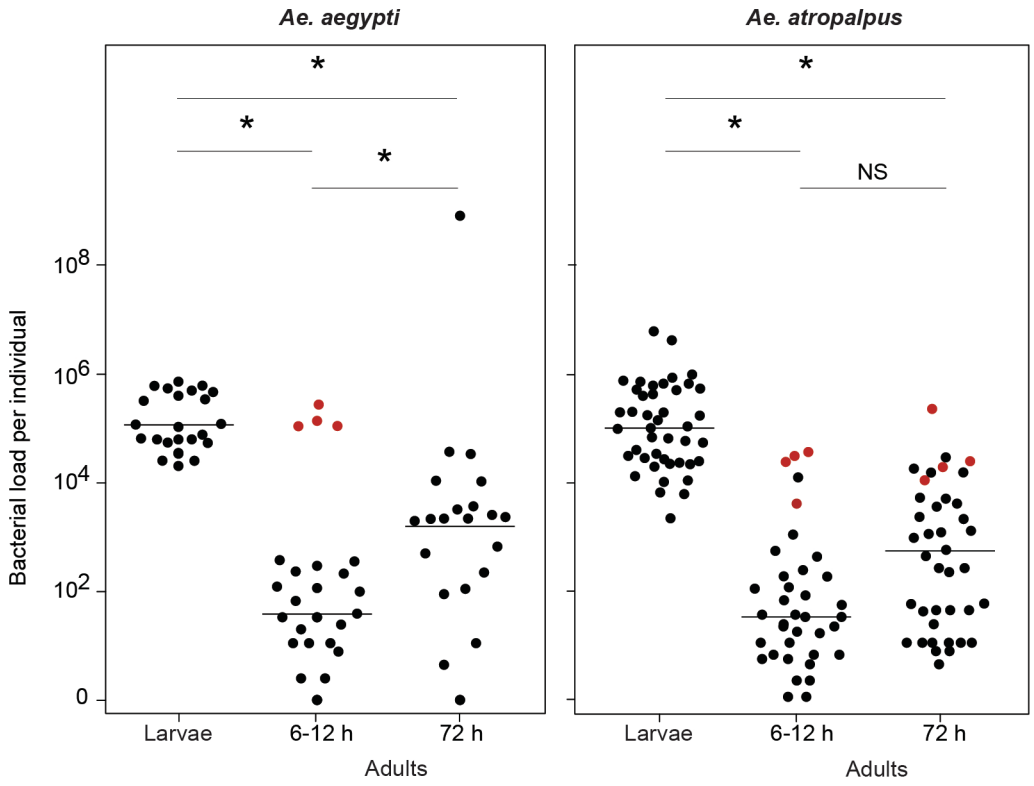


Figure 5.5. Colony-forming unit counts in 24 h old fourth instars (Larvae), 6–12 h adult females, and 72 h adult females. Treatments and the number of individuals analyzed per life stage are the same as in Figure 5.4. For *Aedes aegypti*, bacterial loads significantly differed among treatments in larvae (ANOVA: $F_{(5,18)} = 13.9$, $P < 0.0001$) and 6–12 h adults (ANOVA: $F_{(5,22)} = 19.6$, $P < 0.0001$), but did not differ in 72 h adults (ANOVA: $F_{(5,16)} = 1.6$, $P = 0.21$). Between stage comparisons indicated that bacterial loads significantly differed between larvae, 6–12 h adults and 72 h adults (ANOVA: $F_{(2,71)} = 31.6$, $P < 0.0001$; followed by a Tukey-Kramer HSD test). For *Ae. atropalpus*, bacterial loads significantly differed among treatments in larvae (ANOVA: $F_{(5,34)} = 12.4$, $P < 0.0001$), 6–12 h adults (ANOVA: $F_{(5,33)} = 8.9$, $P < 0.0001$) and 72 h adults (ANOVA: $F_{(5,36)} = 6.8$, $P = 0.0002$). Between stage comparisons indicated that larvae (*) had higher bacterial loads than 6–12 h or 72 h adults, which did not differ from one another (NS) (ANOVA: $F_{(1,118)} = 86.6$, $P < 0.0005$; followed by a Tukey-Kramer HSD test). For *Ae. aegypti* adults (6–12 h) and *Ae. atropalpus* adults (6–12 and 72 h), Dunnett's tests indicated treatment differences were due to higher colony counts for gnotobiotic individuals colonized by *Chryseobacterium* (red data points).

in fourth instars, lowest in 6–12 h adults and intermediate in 72 h adults (Figure 5.5). In *Ae. atropalpus*, bacterial loads were also highest in fourth instars but no difference was detected between 6–12 h and 72 h adults (Figure 5.5).

5.5 Discussion

Aedes aegypti and *Ae. atropalpus* females are similar in size when reared as larvae under the same conditions, but *Ae. aegypti* emerges with lower nutrient reserves as measured by stored glycogen and protein (Telang et al. 2006; Gulia-Nuss et al. 2015). For both species, results in the literature also indicate the nutrients consumed by larvae affect adult size and the average number of eggs per clutch females lay (Briegel 2003; Telang et al. 2006). This finding suggests nutrient based thresholds play a role in egg production (Chambers & Klowden 1994; Telang et al. 2006; Gulia-Nuss et al. 2012; 2015). In effect, *Ae. aegypti* females emerge with insufficient nutrient reserves and enter an arrested previtellogenic state that blood feeding overcomes by providing additional nutrients. *Aedes atropalpus* females in contrast emerge with sufficient nutrients to initiate egg formation without blood feeding. Enhanced nutrient acquisition along with genetic factors has also been implicated in anautogenous versus autogenous reproduction in other species (O'Meara 1985; Merritt et al. 1992; Su & Mulla 1997; Hugo et al. 2003; Mori et al. 2008; Ahmed 2013).

An earlier study used antibiotic treatment to determine whether gut bacteria in adult *Ae. aegypti* affect blood meal digestion (Gaio et al. 2011). Treatment with carbenicillin and tetracycline reduced the abundance of culturable bacteria in the midgut, which correlated with slower breakdown of the blood meal bolus and small, but statistically significant reductions in the number of eggs females laid. Identification of an *Enterobacter* and *Serratia* species with hemolytic activity further supported a role for the gut microbiota in blood meal processing. Outside of this work, however, what role if any the gut microbiota plays in female-associated fitness including egg production is unknown in mosquitoes (Minard et al. 2013), which is why we conducted this study.

Our previous results showed that certain genera of Actinobacteria (*Microbacterium* and *Leucobacter*), Bacteroidetes (*Chryseobacterium* and *Sphingobacterium*), Betaproteobacteria (*Comamonas* and *Aquitalea*) and Firmicutes (*Paenibacillus*) are present in the digestive tracts of both *Ae. aegypti* and *Ae. atropalpus* larvae when reared under identical conditions in the laboratory (Coon et al. 2014). The relative abundance of these community members, however, differs with Bacteroidetes and Actinobacteria predominating in *Ae. aegypti* and Betaproteobacteria predominating in *Ae. atropalpus*. These differences guided our choice of the gut bacteria used in this study to compare the effects individual members have on fitness relative to

conventionally reared individuals with a mixed community of bacteria or axenic larvae with no gut bacteria.

The first part of our study corroborates prior findings by showing that axenic *Ae. aegypti* and *Ae. atropalpus* larvae die as first instars. They also show that *Microbacterium* and *Leucobacter* fail to colonize larvae of either species, which likewise results in no survival. This finding strongly suggests *Microbacterium* and *Leucobacter* present in our conventionally reared larvae require other bacteria to survive in *Ae. aegypti* and *Ae. atropalpus*. The other community members we tested resulted in nearly all *Ae. aegypti* larvae developing into adults that did not differ from adults of conventionally reared larvae in development time, size or the number of mature eggs females produced after consuming a blood meal. In contrast, only the two Betaproteobacteria (*Aquitalea* and *Comamonas*) tested in *Ae. atropalpus* supported survival, development times and adult body sizes that were similar to those of conventionally reared larvae. Only *Ae. atropalpus* larvae colonized by *Comamonas* produced the same number of eggs per first clutch as conventionally reared females. The three other community members we tested (*Paenibacillus*, *Chryseobacterium* and *Sphingobacterium*) are more abundant in conventionally reared *Ae. aegypti* than *Ae. atropalpus*. Each of these *Paenibacillus*, *Chryseobacterium* and *Sphingobacterium* species colonized *Ae. atropalpus* to produce gnotobiotic larvae and increased survival relative to

axenic larvae. However, survival and other fitness measures were also much lower than those of conventionally reared *Ae. atropalpus*.

Taken together, these results indicate that several members of the gut community in *Ae. aegypti* larvae support development and egg production to levels that are comparable to conventionally reared individuals with a mixed bacterial community. They also suggest the differential abundance of these bacteria in conventionally reared *Ae. aegypti* larvae does not reflect the ability of these community members to rescue larval development or egg production by adult females after a blood meal. This is not the case though for *Ae. atropalpus*, where the two Betaproteobacteria we tested are more abundant in conventionally reared larvae and also had a more positive effect on survival, size and egg production in gnotobiotic larvae. Thus, one important element of autogeny in *Ae. atropalpus* could be greater dependence on the composition of the gut microbiota for development and reproduction. In addition, the impacts of gut bacteria on nutrient reserves in *Ae. aegypti* are potentially compensated for by the extra nutrients females obtain from a blood meal.

Gnotobiotic *Ae. atropalpus* colonized by *Comamonas* emerged with similar stores of glycogen and protein but significantly lower total lipid than conventionally reared females. In contrast, gnotobiotic females colonized by *Aquitalea* emerged with lower glycogen, protein, and lipid stores. Reduced nutrient uptake and metabolism in axenic

or gnotobiotic backgrounds have been reported for mice, zebrafish, *Daphnia* and *Drosophila* (Backhed et al. 2004; Ridley et al. 2012; Semova et al. 2012; Newell & Douglas 2014; Sison-Mangus et al. 2015). The gut microbiota of *Drosophila* is also known to promote growth by modulating nutrient sensing through the target of rapamycin and insulin signaling pathways, which intersect to regulate metabolism (Shin et al. 2011; Storelli et al. 2011). Thus, the higher lipid stores in conventionally reared females suggest other community members besides *Aquitalea* or *Comamonas* are required for normal lipid storage. However, lower lipid levels in *Comamonas*-colonized females did not correlate with reduced first clutch sizes, which suggests stored protein is the more important determinant for production of a first clutch. The importance of stored protein may also underlie why gnotobiotic females colonized by *Aquitalea* produced smaller clutches.

Most insects including mosquitoes lay eggs that by dry weight consist primarily of protein but also 30–40% lipid that is predominantly triacylglycerol (TAG) (Attardo et al. 2005; Ziegler & van Antwerpen 2006). TAG is synthesized from free fatty acids and glycerol but insect eggs exhibit little or no de novo fatty acid synthetic activity. Thus, yolk protein and TAG are both imported from the fat body into oocytes during oogenesis (Ziegler & van Antwerpen 2006). In *Ae. aegypti*, digestion of a blood meal primarily provides amino acids, which suggests TAG packaged into eggs during the first

gonadotropic cycle derives primarily from stores in the fat body while yolk protein is produced from a combination of nutrient stores and blood meal derived amino acids (Attardo et al. 2005). In the case of *Ae. atropalpus*, however, TAG and yolk protein must both come from teneral reserves in the fat body. The mobilization of nutrient stores in *Ae. atropalpus* colonized by *Comamonas* is of interest because these females have lower stored lipid relative to females of conventionally reared larvae, but the number of mature eggs produced by both sets of females was similar, as were their protein and carbohydrate stores. How these gnotobiotic females accomplish this is unclear. One possibility is their lower lipid reserves are sufficient to mobilize as TAG and produce a comparable number of mature eggs as conventionally reared females. Alternatively, excess carbohydrate or protein stores may be catabolized to acetyl-CoA, which is the precursor for fatty acid synthesis and thus TAG importation, and would allow females to produce a similar number of mature eggs as conventionally reared females with larger lipid reserves.

Excluding *Microbacterium* and *Leucobacter*, our measures of bacterial abundance in gnotobiotic *Ae. aegypti* and *Ae. atropalpus* showed that the average number of living bacteria per individual was either similar or slightly lower in most gnotobiotic treatments when compared to conventionally reared controls. This finding indicates each of the community members we tested proliferated in the absence of other community

members, but usually did not exceed the total abundance of bacteria in conventional larvae that host a mixed bacterial community. These results indicate that the higher survival and egg production of gnotobiotic *Ae. atropalpus* colonized by *Comamonas* was not due to this bacterium being more abundant in the guts of larvae or adults than the other community members we tested. Thus, other features of this bacterium likely underlie its positive effects for *Ae. atropalpus*. These data also indicate that bacterial abundance in gnotobiotic and conventionally reared individuals is lower in adults than fourth instar larvae, which is a feature previously noted for *Ae. aegypti* (Coon et al. 2014).

5.6 Conclusions

Results of this study show that *Ae. aegypti* and *Ae. atropalpus* larvae both require gut bacteria to develop into adults, but individual gut bacteria differentially affect development and reproduction by both species when reared as gnotobiotic larvae. In particular, autogenous egg production by *Ae. atropalpus* has a greater dependence on the specific members of the gut microbiota, while the added nutrients females obtain from blood feeding potentially make *Ae. aegypti* females less dependent on the composition of the gut microbiota.

5.7 Acknowledgements

The authors thank A. Elliot for assistance with the fecundity and teneral reserve assays. We also thank both A. Elliot and S.R. Robertson for assistance with mosquito colonies. This work was supported by awards from the US National Science Foundation (Graduate Research Fellowship 038550–04) (KLC) and National Institutes of Health (R01AI06892 and T32GM007103) (MRS).

CHAPTER 6

CONCLUSIONS

The predominance of heritable bacterial endosymbionts in insects has led several researchers to propose the concept that hosts and their associated microbes should be treated as a single, integrated unit (i.e. holobiont). However, recent advances in sequencing technologies have revealed more complex associations between insects and environmentally acquired microbes, including gut bacteria. The promiscuous lifestyle of many gut bacteria has important implications for our understanding of how host-symbiont associations evolve. Here, I integrated innovative experimental and computational approaches to show that mosquitoes require living bacteria in their gut to develop. Furthermore, my results demonstrate that mosquitoes do not rely on a

particular species or community of bacteria, but rather have evolved to depend on a developmental cue provided by most bacteria in their environment. These results provide novel insights into insect-microbe interactions that could be applied to other aquatic insects with similar life histories. Importantly, this research also offers insights for measures to control vector species of mosquitoes and the pathogens they transmit. Specifically, results from this work identify bacteria-mediated hypoxia as an essential signal in mosquito development. Future work will characterize the mechanism by which this signal is transduced in the mosquito gut and reveal critical host pathways that may be amenable to disruption for mosquito control.

APPENDIX A

SUPPORTING INFORMATION FOR CHAPTER 2

A.1 Supplementary Tables

Table A.1: Primers designed and used during the study.

*Table shown on next page

Table A.1 (continued)

Name	Sequence	Target gene	Target	Tm (°C)	Reference
General 16S primers					
27F	GAGAGTTTGATCCTGGCTCAG	16S rRNA	Most bacteria	50	Lane 1991
1492R	GGTTACCTTGTTACGACTT	16S rRNA	Most bacteria	50	Lane 1991
Genus-specific primers					
WspF	TGGTCCAATAAGTGATGAAGAAACTAGC TA	16S rRNA	*Wolbachia* spp.	65-55	Jeyaprakash & Hoy 2000
WspR	AAAAATTAAACGCTACTCCAGCTTCTGC AC	16S rRNA	*Wolbachia* spp.	65-55	Jeyaprakash & Hoy 2000
Acine1	ACTTTAAGCGAGGAGGAGGCT	*rrs*	*Acinetobacter* spp.	63	Zouache et al. 2009
Ac	GCGCCACTAAAGCCTCAAAGGCC	*rrs*	*Acinetobacter* spp.	63	Zouache et al. 2009
Aero-F	GGCCTTGACATGTCTGGAAT	16S rRNA	*Aeromonas* spp.	55	
Aero-R	CCCTTGAGTTCCCACCATTA	16S rRNA	*Aeromonas* spp.	55	
Aqui-F	GCACAGCTAGAGTGCGTCAG	16S rRNA	*Aquitalea* spp.	55	
Aqui-R	GGTCAACTTCTCGCGTTAGC	16S rRNA	*Aquitalea* spp.	55	
CH45F	GGCCTAACACATGCAAGC	16S rRNA	*Chryseobacterium* spp.	66	Green et al. 2007
CH1112R	GCAACTAGTGACAGGGG	16S rRNA	*Chryseobacterium* spp.	66	Green et al. 2007
Micro-F	GTTGTGGGGTCCATTCCACG	16S rRNA	*Microbacterium* spp.	70	Bora 2011
Micro-R	TACCGATCCCACCTTCGACG	16S rRNA	*Microbacterium* spp.	70	Bora 2011
843F	TCGATACCCTTGGTGCCGAAGT	16S rRNA	*Paenibacillus* spp.	58	Priest 2009
1484R	TACCTTGTTACGACTTCACCCCA	16S rRNA	*Paenibacillus* spp.	58	Priest 2009
Tecol-754	CAGTACAGGTAGACTTCTG	*tuf*	*Escherichia coli*	58	Maheaux et al. 2009
Tecol-553	TGGGAAGCGAAAATCCTG	*tuf*	*E. coli*	58	Maheaux et al. 2009
Pyrotagging primers					
27Fshort-B	gcAACACGAGTTTGATCMTGGCTCA	16S rRNA	Most bacteria	55	modified from Martinson et al. 2011
27Fshort-C	catCGGTTGAGTTTGATCMTGGCTCA	16S rRNA	Most bacteria	55	modified from Martinson et al. 2011
27Fshort-D	agctGTCAAGAGTTTGATCMTGGCTCA	16S rRNA	Most bacteria	55	modified from Martinson et al. 2011
27Fshort-E	gAAGCGGAGTTTGATCMTGGCTCA	16S rRNA	Most bacteria	55	modified from Martinson et al. 2011
27Fshort-F	tgCCACAGAGTTTGATCMTGGCTCA	16S rRNA	Most bacteria	55	modified from Martinson et al. 2011
342R-2	agAGTGGCTGCTGCSYCCCGTAG	16S rRNA	Most bacteria	55	modified from Kunin et al. 2010
342R-3	tcaACGTCCTGCTGCSYCCCGTAG	16S rRNA	Most bacteria	55	modified from Kunin et al. 2010
342R-4	gttcTCAGCCTGCTGCSYCCCGTAG	16S rRNA	Most bacteria	55	modified from Kunin et al. 2010
342R-5	aCTAGGCTGCTGCSYCCCGTAG	16S rRNA	Most bacteria	55	modified from Kunin et al. 2010
342R-6	tcgGCTTACTGCTGCSYCCCGTAG	16S rRNA	Most bacteria	55	modified from Kunin et al. 2010

Table A.2: Bacterial taxa isolated, cultured and identified from *Aedes aegypti*.

Life stage isolate was collected from	**Colony color (morphology)**	**Culture medium**	**Genus**	**No. of isolates**
Larvae	Tan-buff (medium, circular, convex, smooth)	Blood agar (TSA base)	*Aeromonas*	2
Larvae	Yellow-green (small, circular, convex, smooth)	Nutrient agar (BHI)	*Aquitalea*	1
Larvae	Orange (small, circular, convex, smooth)	Nutrient agar (BHI)	*Chryseobacterium*	2
Larvae	Yellow-tan (medium, circular, convex, smooth)	Blood agar (TSA base)	*Enterobacter*	1
Larvae	Yellow (pinpoint, circular, convex, smooth)	Nutrient agar (BHI; 0.5% glucose)	*Flavobacterium*	1
Larvae	Pink (small, circular, convex, viscid)	Nutrient agar (20% BHI)	*Flectobacilus*	1
Larvae	White (<0.1mm, circular, convex, smooth)	Blood agar (TSA base)	*Microbacterium*	1
Larvae	White-pink (small, circular, slightly umbonate)	Nutrient agar (BHI)	*Paenibacillus*	2
Larvae	White (medium, circular, flat, mucoid)	Nutrient agar (BHI)	*Pseudomonas*	1
Larvae	White-yellow (small, circular, convex, smooth)	Nutrient agar (BHI)	*Sphingobacterium*	2
NBF adults	White (small, circular, convex, slightly opaque)	Nutrient agar (BHI; 0.5% glucose)	*Acinetobacter*	1
NBF adults	Orange (small, circular, convex, smooth)	Nutrient agar (BHI; 0.5% glucose)	*Chryseobacterium*	1
BF adults	White (small, circular, mucoid, slightly opaque)	Nutrient agar (TSA)	*Serratia*	3
Eggs	White (small, circular, convex, slightly opaque)	Nutrient agar (BHI; 0.5% NaCl)	*Acinetobacter*	1
Eggs	Yellow-tan (medium, circular, convex, smooth)	Nutrient agar (BHI; 0.5% glucose)	*Enterobacter*	1
Eggs	White (small, circular, convex, viscid, opaque)	Nutrient agar (BHI)	*Staphylococcus*	1

BHI: 12.5 g BHI + 7.5 g agar / 500 ml water
TSA: 20 g TSA + 7.5 g agar / 500 ml water
Blood agar: TSA + 5% sheep blood

A.2 Supplementary Figures

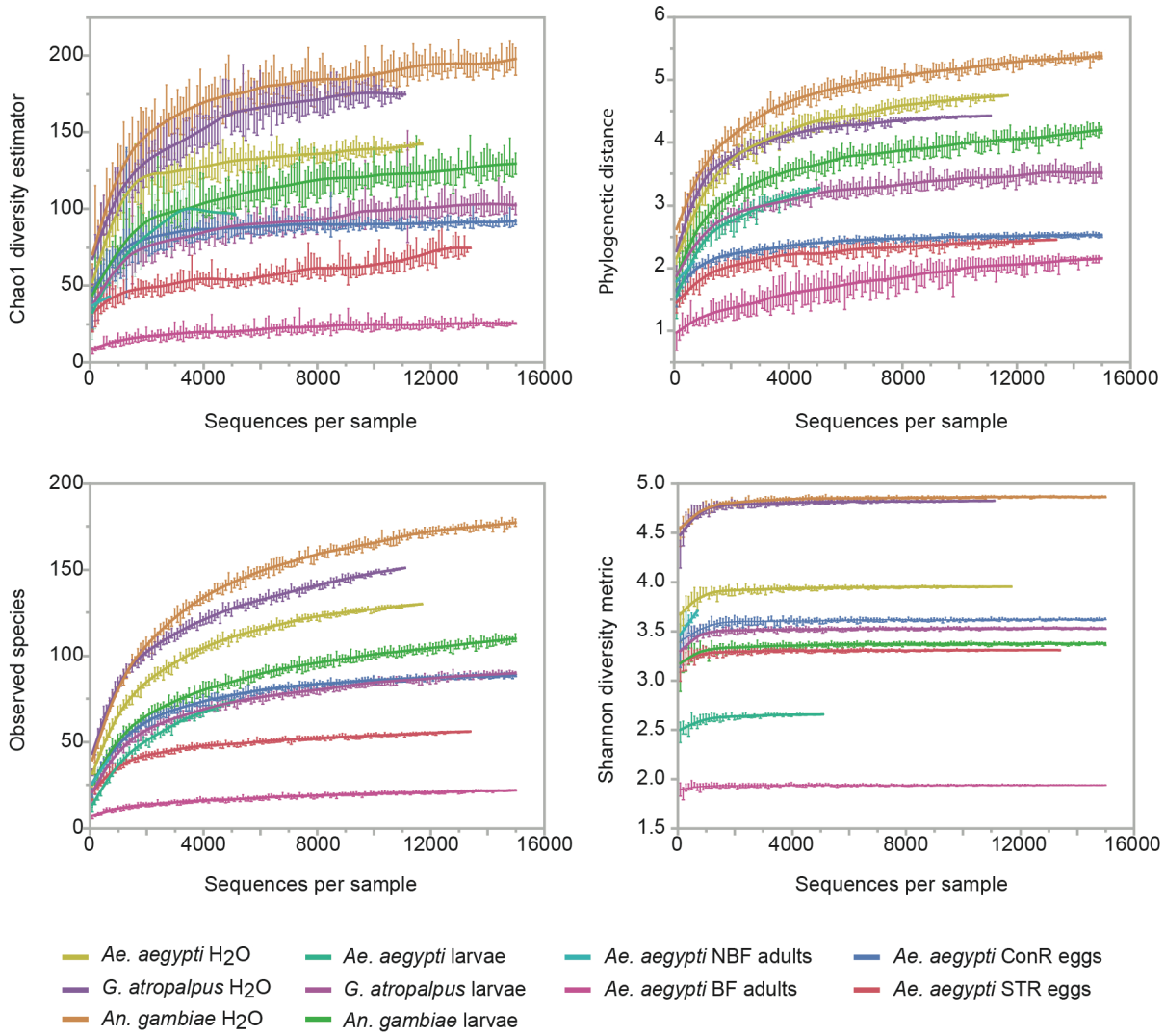


Figure A.1: Rarefaction data from pyrotagging experiments. Reads were sampled starting at 100 sequences/step and increased in increments of 50 until the total number of reads for a sample had been reached. Each step was sampled 10 times. Lines represent smoothed means across each increment. Error bars represent the 95% confidence interval of the mean of the 10 samples.

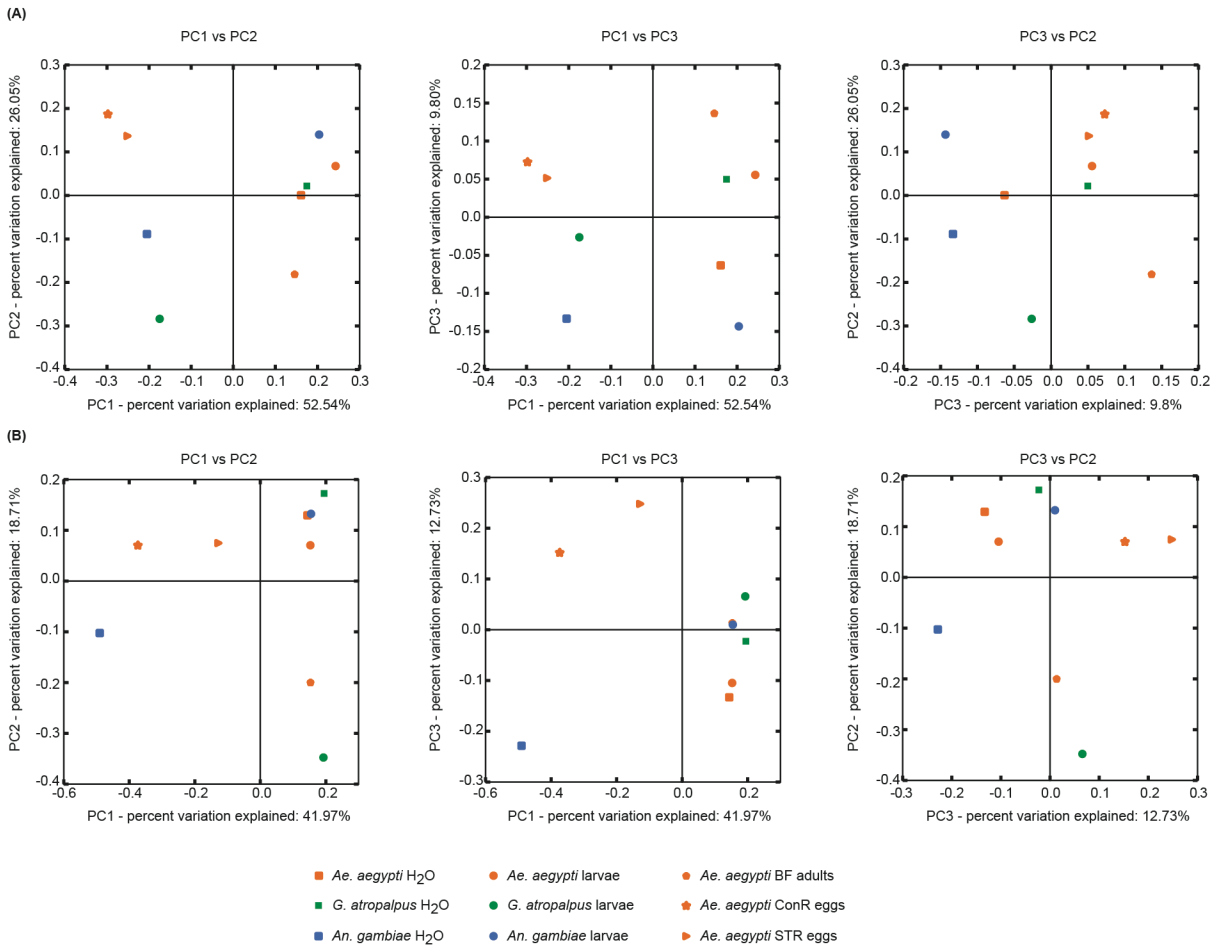


Figure A.2: Principal components analysis of (A) weighted and (B) unweighted UniFrac distances of the samples. There were not enough sequences in the *Aedes aegypti* NBF sample to include in the analysis.

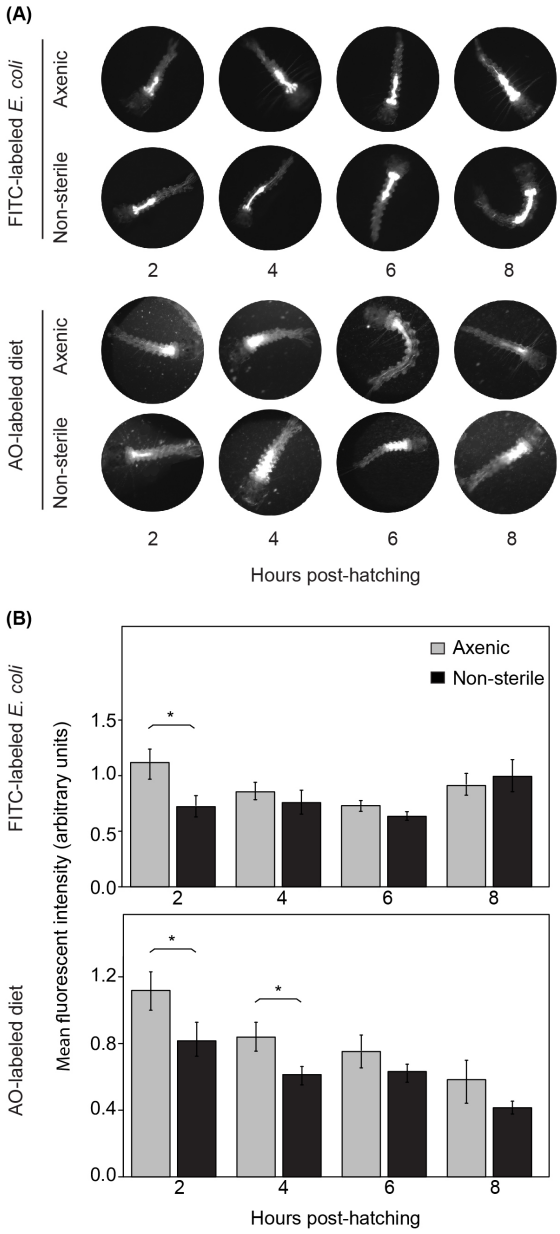


Figure A.3: Axenic and non-sterile *Aedes aegypti* larvae consume dead bacteria and diet. (A) Epifluorescent images of individual larvae 2-8 h after placing FITC-labeled heat killed *Escherichia coli* or AO-labeled diet into culture flasks. Note that fluorescent tracer is visible in the gastric caecae of each larva as early as 2 h after the bioassay began. (B) Mean fluorescent intensity in larvae 2-8 h after placement of FITC-labeled *E. coli* or AO-labeled diet with larvae. Columns present mean values with 95% confidence intervals for each treatment. T-tests were conducted separately for each time point. Axenic larvae consumed more bacteria or diet than non-sterile larvae after 2 h or 4 h (*, $p < 0.01$). However, no differences were detected at the 6 and 8 h time points. A minimum of 10 larvae was measured per treatment and time point.

APPENDIX B

SUPPORTING INFORMATION FOR CHAPTER 3

B.1 Supplementary Methods

B.1.1: Surface sterilization of eggs

Axenic larvae were produced by placing eggs into sterile Petri dishes containing 70% EtOH for 5 min, transferring to a solution of 3% bleach and 0.1% ROCCAL-D (Pfizer) for 3 min, transferring again to 70% EtOH for 5 min, and rinsing three times in sterile water. Sterile water was produced by autoclaving.

B.1.2: Verification of sterility of axenic larvae and diet

Sterility of larvae was confirmed by culture-based and PCR analysis. A pool of 30 first instars from surface sterilized eggs were rinsed in sterile water and transferred to 100 µl of sterile phosphate buffered saline and homogenized. Larval homogenates were plated on Luria Broth (LB) and brain-heart infusion (BHI) agar plates at 28 °C for 24-72 h and subsequently checked for colonies. DNA was also isolated from three pools of 10 larvae using the Gentra Puregene Yeast/Bacteria Kit (Qiagen). Following DNA extraction, the 16S rDNA gene was amplified using either universal bacterial primers 27F (5'-GAGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-GGTTACCTTGTTACGACTT-3') or *Wolbachia*–specifc primers WspF (5'-TGGTCCAATAAGTGATGAAGAAACTAGCTA-3') and WspR (5'-

AAAAATTAAACGCTACTCCAGCTTCTGCAC-3'). For each mosquito species, three replicate 10 µl reactions were conducted, each containing 1 x HotMaster Taq Buffer, 200 µM of each dNTP, 0.2 µM of each primer, 1 U of Hotmaster Taq polymerase (5 Prime) and 0.8 µl of DNA template. Reactions were run on a MJ Mini thermocycler (Bio-Rad) with amplification cycle conditions as follows: denaturation at 94 °C for 2 min, followed by 30 cycles of 94 °C for 20 s, 50 °C for 20 s, and 65 °C for 1 min, and final extension at 65 °C for 5 min. Products from the three replicate amplifications were pooled and 5 µl of the combined PCR product was electrophoresed on a 1% (wt/vol) agarose gel. The same methods were used to verify sterility of irradiated diet.

B.2 Supplementary Tables

Table B.1: Barcode sequences for water and mosquito libraries.

*Table shown on next page

Table B.1 (continued)

Technical replicate	Site	Sampling date	Sample source	Mosquito species	Sample ID	Forward 16S	Reverse 16S
1	Site 1	6/23/14	Larvae	*Aedes aegypti*	24	CAACACCCTACGGG[ATCG]GGC[AT]GCAG	GGATTAGATACCC[CGT]|AGT]GTAGTCGATTGAGA
1	Site 1	6/23/14	Larvae	*Culex quinquefasciatus*	23	CAACACCCTACGGG[ATCG]GGC[AT]GCAG	GGATTAGATACCC[CGT]|AGT]GTAGTCGGAATCG
1	Site 1	6/23/14	Water	NA	37	TCGGTCAACCTACGGG[ATCG]GGC[AT]GCAG	GGATTAGATACCC[CGT]|AGT]GTAGTCTTCCT
1	Site 1	7/21/14	Larvae	*Ae. aegypti*	29	ATCGGTTCCTACGGG[ATCG]GGC[AT]GCAG	GGATTAGATACCC[CGT]|AGT]GTAGTCCCTAG
1	Site 1	7/21/14	Larvae	*C. quinquefasciatus*	27	ATCGGTTCCTACGGG[ATCG]GGC[AT]GCAG	GGATTAGATACCC[CGT]|AGT]GTAGTCGACGTGG
1	Site 1	7/21/14	Water	NA	28	ATCGGTTCCTACGGG[ATCG]GGC[AT]GCAG	GGATTAGATACCC[CGT]|AGT]GTAGTCGCTGAGAA
1	Site 1	9/15/14	Larvae	*Ae. aegypti*	22	CAACACCCTACGGG[ATCG]GGC[AT]GCAG	GGATTAGATACCC[CGT]|AGT]GTAGTCAGTCTC
1	Site 1	9/15/14	Water	NA	19	CAACACCCTACGGG[ATCG]GGC[AT]GCAG	GGATTAGATACCC[CGT]|AGT]GTAGTCACTTCGC
1	Site 2	6/23/14	Larvae	*Ae. aegypti*	31	ATCGGTTCCTACGGG[ATCG]GGC[AT]GCAG	GGATTAGATACCC[CGT]|AGT]GTAGTCACTTCGC
1	Site 2	6/23/14	Water	NA	41	TCGGTCAACCTACGGG[ATCG]GGC[AT]GCAG	GGATTAGATACCC[CGT]|AGT]GTAGTCCCTAG
1	Site 2	9/15/14	Larvae	*Ae. aegypti*	34	ATCGGTTCCTACGGG[ATCG]GGC[AT]GCAG	GGATTAGATACCC[CGT]|AGT]GTAGTCAGTCTC
1	Site 2	9/15/14	Water	NA	33	ATCGGTTCCTACGGG[ATCG]GGC[AT]GCAG	GGATTAGATACCC[CGT]|AGT]GTAGTCCAGAT
1	Site 3	7/21/14	Larvae	*Ae. aegypti*	20	CAACACCCTACGGG[ATCG]GGC[AT]GCAG	GGATTAGATACCC[CGT]|AGT]GTAGTCATAGGATT
1	Site 3	7/21/14	Water	NA	38	TCGGTCAACCTACGGG[ATCG]GGC[AT]GCAG	GGATTAGATACCC[CGT]|AGT]GTAGTCCCACTC
1	Site 3	9/15/14	Larvae	*Ae. aegypti*	36	ATCGGTTCCTACGGG[ATCG]GGC[AT]GCAG	GGATTAGATACCC[CGT]|AGT]GTAGTCGATTGAGA
1	Site 3	9/15/14	Water	NA	40	TCGGTCAACCTACGGG[ATCG]GGC[AT]GCAG	GGATTAGATACCC[CGT]|AGT]GTAGTCGCTGAGAA
1	Site 4	7/21/14	Larvae	*Ae. aegypti*	35	ATCGGTTCCTACGGG[ATCG]GGC[AT]GCAG	GGATTAGATACCC[CGT]|AGT]GTAGTCGGAATCG
1	Site 4	7/21/14	Water	NA	21	CAACACCCTACGGG[ATCG]GGC[AT]GCAG	GGATTAGATACCC[CGT]|AGT]GTAGTCCAGAT
1	Site 4	9/15/14	Larvae	*Ae. aegypti*	25	ATCGGTTCCTACGGG[ATCG]GGC[AT]GCAG	GGATTAGATACCC[CGT]|AGT]GTAGTCTTCCT
1	Site 4	9/15/14	Water	NA	32	ATCGGTTCCTACGGG[ATCG]GGC[AT]GCAG	GGATTAGATACCC[CGT]|AGT]GTAGTCATAGGATT
1	Site 5	7/21/14	Larvae	*C. quinquefasciatus*	39	TCGGTCAACCTACGGG[ATCG]GGC[AT]GCAG	GGATTAGATACCC[CGT]|AGT]GTAGTCGACGTGG
1	Site 5	7/21/14	Water	NA	30	ATCGGTTCCTACGGG[ATCG]GGC[AT]GCAG	GGATTAGATACCC[CGT]|AGT]GTAGTCTAAGCA
1	Site 5	9/15/14	Larvae	*C. quinquefasciatus*	16	CAACACCCTACGGG[ATCG]GGC[AT]GCAG	GGATTAGATACCC[CGT]|AGT]GTAGTCGCTGAGAA
1	Site 5	9/15/14	Water	NA	26	ATCGGTTCCTACGGG[ATCG]GGC[AT]GCAG	GGATTAGATACCC[CGT]|AGT]GTAGTCCCACTC
1	Athens, GA	7/7/14	Larvae	*Ae. albopictus*	3	TGATTGACCCTACGGG[ATCG]GGC[AT]GCAG	GGATTAGATACCC[CGT]|AGT]GTAGTCAGTCTC
1	Athens, GA	7/7/14	Larvae	*C. quinquefasciatus*	2	TGATTGACCCTACGGG[ATCG]GGC[AT]GCAG	GGATTAGATACCC[CGT]|AGT]GTAGTCGGAATCG
1	Athens, GA	7/7/14	Water	NA	6	GGTACCCTACGGG[ATCG]GGC[AT]GCAG	GGATTAGATACCC[CGT]|AGT]GTAGTCTAAGCA
1	Athens, GA	9/23/14	Larvae	*Ae. albopictus*	1	TGATTGACCCTACGGG[ATCG]GGC[AT]GCAG	GGATTAGATACCC[CGT]|AGT]GTAGTCGATTGAGA
1	Athens, GA	9/23/14	Larvae	*C. quinquefasciatus*	4	GGTACCCTACGGG[ATCG]GGC[AT]GCAG	GGATTAGATACCC[CGT]|AGT]GTAGTCGCTGAGAA
1	Athens, GA	9/23/14	Water	NA	18	CAACACCCTACGGG[ATCG]GGC[AT]GCAG	GGATTAGATACCC[CGT]|AGT]GTAGTCTAAGCA
1	Laboratory	9/30/14	Larvae	*Ae. aegypti*	17	CAACACCCTACGGG[ATCG]GGC[AT]GCAG	GGATTAGATACCC[CGT]|AGT]GTAGTCCCTAG
1	Laboratory	9/30/14	Larvae	*Ae. albopictus*	15	CAACACCCTACGGG[ATCG]GGC[AT]GCAG	GGATTAGATACCC[CGT]|AGT]GTAGTCGACGTGG
1	Laboratory	9/30/14	Larvae	*C. quinquefasciatus*	12	GGTACCCTACGGG[ATCG]GGC[AT]GCAG	GGATTAGATACCC[CGT]|AGT]GTAGTCGATTGAGA
1	Laboratory	9/30/14	Water	*Ae. aegypti*	10	GGTACCCTACGGG[ATCG]GGC[AT]GCAG	GGATTAGATACCC[CGT]|AGT]GTAGTCAGTCTC
1	Laboratory	9/30/14	Water	*Ae. albopictus*	9	GGTACCCTACGGG[ATCG]GGC[AT]GCAG	GGATTAGATACCC[CGT]|AGT]GTAGTCCAGAT
1	Laboratory	9/30/14	Water	*C. quinquefasciatus*	8	GGTACCCTACGGG[ATCG]GGC[AT]GCAG	GGATTAGATACCC[CGT]|AGT]GTAGTCATAGGATT
2	Site 1	6/23/14	Larvae	*Ae. aegypti*	24	CTGGATGCCTACGGG[ATCG]GGC[AT]GCAG	GGATTAGATACCC[CGT]|AGT]GTAGTCTTCCT
2	Site 1	6/23/14	Larvae	*C. quinquefasciatus*	23	GCCACACCTACGGG[ATCG]GGC[AT]GCAG	GGATTAGATACCC[CGT]|AGT]GTAGTCGATTGAGA
2	Site 1	6/23/14	Water	NA	37	GGTACCCTACGGG[ATCG]GGC[AT]GCAG	GGATTAGATACCC[CGT]|AGT]GTAGTCGGAATCG
2	Site 1	7/21/14	Larvae	*Ae. aegypti*	29	CTGGATGCCTACGGG[ATCG]GGC[AT]GCAG	GGATTAGATACCC[CGT]|AGT]GTAGTCTAAGCA
2	Site 1	7/21/14	Larvae	*C. quinquefasciatus*	27	CTGGATGCCTACGGG[ATCG]GGC[AT]GCAG	GGATTAGATACCC[CGT]|AGT]GTAGTCGCTGAGAA
2	Site 1	7/21/14	Water	NA	28	CTGGATGCCTACGGG[ATCG]GGC[AT]GCAG	GGATTAGATACCC[CGT]|AGT]GTAGTCCCTAG
2	Site 1	9/15/14	Larvae	*Ae. aegypti*	22	GCCACACCTACGGG[ATCG]GGC[AT]GCAG	GGATTAGATACCC[CGT]|AGT]GTAGTCGGAATCG
2	Site 1	9/15/14	Water	NA	19	GCCACACCTACGGG[ATCG]GGC[AT]GCAG	GGATTAGATACCC[CGT]|AGT]GTAGTCATAGGATT
2	Site 2	6/23/14	Larvae	*Ae. aegypti*	31	CTGGATGCCTACGGG[ATCG]GGC[AT]GCAG	GGATTAGATACCC[CGT]|AGT]GTAGTCATAGGATT
2	Site 2	6/23/14	Water	NA	41	TGATTGACCCTACGGG[ATCG]GGC[AT]GCAG	GGATTAGATACCC[CGT]|AGT]GTAGTCTAAGCA

Technical replicate	Site	Sampling date	Sample source	Mosquito species	Sample ID	Forward 16S	Reverse 16S
Continued from the previous page...							
2	Site 2	9/15/14	Larvae	*Ae. aegypti*	34	CTGGATGCCTACGGG[ATCG]GGC[AT]GCAG	GGATTAGATACCC[CGT][AGT]GTAGTCGGAATCG
2	Site 2	9/15/14	Water	NA	33	GGTACCCTACGGG[ATCG]GGC[AT]GCAG	GGATTAGATACCC[CGT][AGT]GTAGTCAGTCTC
2	Site 3	7/21/14	Larvae	*Ae. aegypti*	20	GGTACCCTACGGG[ATCG]GGC[AT]GCAG	GGATTAGATACCC[CGT][AGT]GTAGTCACTTCGC
2	Site 3	7/21/14	Water	NA	38	TGATTGACCCTACGGG[ATCG]GGC[AT]GCAG	GGATTAGATACCC[CGT][AGT]GTAGTCGACGTGG
2	Site 3	9/15/14	Larvae	*Ae. aegypti*	36	TGATTGACCCTACGGG[ATCG]GGC[AT]GCAG	GGATTAGATACCC[CGT][AGT]GTAGTCTTCCT
2	Site 3	9/15/14	Water	NA	40	TGATTGACCCTACGGG[ATCG]GGC[AT]GCAG	GGATTAGATACCC[CGT][AGT]GTAGTCCCTAG
2	Site 4	7/21/14	Larvae	*Ae. aegypti*	35	CTGGATGCCTACGGG[ATCG]GGC[AT]GCAG	GGATTAGATACCC[CGT][AGT]GTAGTCGATTGAGA
2	Site 4	7/21/14	Water	NA	21	GGTACCCTACGGG[ATCG]GGC[AT]GCAG	GGATTAGATACCC[CGT][AGT]GTAGTCATAGGATT
2	Site 4	9/15/14	Larvae	*Ae. aegypti*	25	CTGGATGCCTACGGG[ATCG]GGC[AT]GCAG	GGATTAGATACCC[CGT][AGT]GTAGTCCCACTC
2	Site 4	9/15/14	Water	NA	32	CTGGATGCCTACGGG[ATCG]GGC[AT]GCAG	GGATTAGATACCC[CGT][AGT]GTAGTCCAGAT
2	Site 5	7/21/14	Larvae	*C. quinquefasciatus*	39	TGATTGACCCTACGGG[ATCG]GGC[AT]GCAG	GGATTAGATACCC[CGT][AGT]GTAGTCGCTGAGAA
2	Site 5	7/21/14	Water	NA	30	GGTACCCTACGGG[ATCG]GGC[AT]GCAG	GGATTAGATACCC[CGT][AGT]GTAGTCCAGAT
2	Site 5	9/15/14	Larvae	*C. quinquefasciatus*	16	GCCACACCTACGGG[ATCG]GGC[AT]GCAG	GGATTAGATACCC[CGT][AGT]GTAGTCCCTAG
2	Site 5	9/15/14	Water	NA	26	CTGGATGCCTACGGG[ATCG]GGC[AT]GCAG	GGATTAGATACCC[CGT][AGT]GTAGTCGACGTGG
2	Athens, GA	7/7/14	Larvae	*Ae. albopictus*	3	AAGCGCCTACGGG[ATCG]GGC[AT]GCAG	GGATTAGATACCC[CGT][AGT]GTAGTCGCTGAGAA
2	Athens, GA	7/7/14	Larvae	*C. quinquefasciatus*	2	AAGCGCCTACGGG[ATCG]GGC[AT]GCAG	GGATTAGATACCC[CGT][AGT]GTAGTCGACGTGG
2	Athens, GA	7/7/14	Water	NA	6	AAGCGCCTACGGG[ATCG]GGC[AT]GCAG	GGATTAGATACCC[CGT][AGT]GTAGTCACTTCGC
2	Athens, GA	9/23/14	Larvae	*Ae. albopictus*	1	AAGCGCCTACGGG[ATCG]GGC[AT]GCAG	GGATTAGATACCC[CGT][AGT]GTAGTCCCACTC
2	Athens, GA	9/23/14	Larvae	*C. quinquefasciatus*	4	AAGCGCCTACGGG[ATCG]GGC[AT]GCAG	GGATTAGATACCC[CGT][AGT]GTAGTCCCTAG
2	Athens, GA	9/23/14	Water	NA	18	GCCACACCTACGGG[ATCG]GGC[AT]GCAG	GGATTAGATACCC[CGT][AGT]GTAGTCACTTCGC
2	Laboratory	9/30/14	Larvae	*Ae. aegypti*	17	GCCACACCTACGGG[ATCG]GGC[AT]GCAG	GGATTAGATACCC[CGT][AGT]GTAGTCTAAGCA
2	Laboratory	9/30/14	Larvae	*Ae. albopictus*	15	GCCACACCTACGGG[ATCG]GGC[AT]GCAG	GGATTAGATACCC[CGT][AGT]GTAGTCGCTGAGAA
2	Laboratory	9/30/14	Larvae	*C. quinquefasciatus*	12	GCCACACCTACGGG[ATCG]GGC[AT]GCAG	GGATTAGATACCC[CGT][AGT]GTAGTCTTCCT
2	Laboratory	9/30/14	Water	*Ae. aegypti*	10	AAGCGCCTACGGG[ATCG]GGC[AT]GCAG	GGATTAGATACCC[CGT][AGT]GTAGTCGGAATCG
2	Laboratory	9/30/14	Water	*Ae. albopictus*	9	AAGCGCCTACGGG[ATCG]GGC[AT]GCAG	GGATTAGATACCC[CGT][AGT]GTAGTCAGTCTC
2	Laboratory	9/30/14	Water	*C. quinquefasciatus*	8	AAGCGCCTACGGG[ATCG]GGC[AT]GCAG	GGATTAGATACCC[CGT][AGT]GTAGTCCAGAT
3	Site 1	6/23/14	Larvae	*Ae. aegypti*	24	GCCACACCTACGGG[ATCG]GGC[AT]GCAG	GGATTAGATACCC[CGT][AGT]GTAGTCTAAGCA
3	Site 1	6/23/14	Larvae	*C. quinquefasciatus*	23	GCCACACCTACGGG[ATCG]GGC[AT]GCAG	GGATTAGATACCC[CGT][AGT]GTAGTCACTTCGC
3	Site 1	6/23/14	Water	NA	37	AAGCGCCTACGGG[ATCG]GGC[AT]GCAG	GGATTAGATACCC[CGT][AGT]GTAGTCGCTGAGAA
3	Site 1	7/21/14	Larvae	*Ae. aegypti*	29	AAGCGCCTACGGG[ATCG]GGC[AT]GCAG	GGATTAGATACCC[CGT][AGT]GTAGTCGATTGAGA
3	Site 1	7/21/14	Larvae	*C. quinquefasciatus*	27	GCCACACCTACGGG[ATCG]GGC[AT]GCAG	GGATTAGATACCC[CGT][AGT]GTAGTCCCACTC
3	Site 1	7/21/14	Water	NA	28	GCCACACCTACGGG[ATCG]GGC[AT]GCAG	GGATTAGATACCC[CGT][AGT]GTAGTCTTCCT
3	Site 1	9/15/14	Larvae	*Ae. aegypti*	22	GCCACACCTACGGG[ATCG]GGC[AT]GCAG	GGATTAGATACCC[CGT][AGT]GTAGTCATAGGATT
3	Site 1	9/15/14	Water	NA	19	CTGGATGCCTACGGG[ATCG]GGC[AT]GCAG	GGATTAGATACCC[CGT][AGT]GTAGTCTTCCT
3	Site 2	6/23/14	Larvae	*Ae. aegypti*	31	AAGCGCCTACGGG[ATCG]GGC[AT]GCAG	GGATTAGATACCC[CGT][AGT]GTAGTCAGTCTC
3	Site 2	6/23/14	Water	NA	41	TCGGTCAACCTACGGG[ATCG]GGC[AT]GCAG	GGATTAGATACCC[CGT][AGT]GTAGTCGATTGAGA
3	Site 2	9/15/14	Larvae	*Ae. aegypti*	34	AAGCGCCTACGGG[ATCG]GGC[AT]GCAG	GGATTAGATACCC[CGT][AGT]GTAGTCACTTCGC
3	Site 2	9/15/14	Water	NA	33	AAGCGCCTACGGG[ATCG]GGC[AT]GCAG	GGATTAGATACCC[CGT][AGT]GTAGTCATAGGATT
3	Site 3	7/21/14	Larvae	*Ae. aegypti*	20	GCCACACCTACGGG[ATCG]GGC[AT]GCAG	GGATTAGATACCC[CGT][AGT]GTAGTCGATTGAGA
3	Site 3	7/21/14	Water	NA	38	AAGCGCCTACGGG[ATCG]GGC[AT]GCAG	GGATTAGATACCC[CGT][AGT]GTAGTCGACGTGG
3	Site 3	9/15/14	Larvae	*Ae. aegypti*	36	AAGCGCCTACGGG[ATCG]GGC[AT]GCAG	GGATTAGATACCC[CGT][AGT]GTAGTCCCTAG
3	Site 3	9/15/14	Water	NA	40	AAGCGCCTACGGG[ATCG]GGC[AT]GCAG	GGATTAGATACCC[CGT][AGT]GTAGTCTTCCT
3	Site 4	7/21/14	Larvae	*Ae. aegypti*	35	AAGCGCCTACGGG[ATCG]GGC[AT]GCAG	GGATTAGATACCC[CGT][AGT]GTAGTCTAAGCA
3	Site 4	7/21/14	Water	NA	21	GCCACACCTACGGG[ATCG]GGC[AT]GCAG	GGATTAGATACCC[CGT][AGT]GTAGTCGGAATCG
3	Site 4	9/15/14	Larvae	*Ae. aegypti*	25	GCCACACCTACGGG[ATCG]GGC[AT]GCAG	GGATTAGATACCC[CGT][AGT]GTAGTCCCTAG
3	Site 4	9/15/14	Water	NA	32	AAGCGCCTACGGG[ATCG]GGC[AT]GCAG	GGATTAGATACCC[CGT][AGT]GTAGTCCAGAT
3	Site 5	7/21/14	Larvae	*C. quinquefasciatus*	39	AAGCGCCTACGGG[ATCG]GGC[AT]GCAG	GGATTAGATACCC[CGT][AGT]GTAGTCCCACTC
3	Site 5	7/21/14	Water	NA	30	AAGCGCCTACGGG[ATCG]GGC[AT]GCAG	GGATTAGATACCC[CGT][AGT]GTAGTCGGAATCG

Technical replicate	Site	Sampling date	Sample source	Mosquito species	Sample ID	Forward 16S	Reverse 16S
Continued from the previous page...							
3	Site 5	9/15/14	Larvae	*C. quinquefasciatus*	16	CTGGATGCCTACGGG[ATCG]GGC[AT]GCAG	GGATTAGATACCC[CGT][AGT]GTAGTCGCTGAGAA
3	Site 5	9/15/14	Water	NA	26	GCCACACCTACGGG[ATCG]GGC[AT]GCAG	GGATTAGATACCC[CGT][AGT]GTAGTCGCTGAGAA
3	Athens, GA	7/7/14	Larvae	*Ae. albopictus*	3	TGATTGACCCTACGGG[ATCG]GGC[AT]GCAG	GGATTAGATACCC[CGT][AGT]GTAGTCATAGGATT
3	Athens, GA	7/7/14	Larvae	*C. quinquefasciatus*	2	TGATTGACCCTACGGG[ATCG]GGC[AT]GCAG	GGATTAGATACCC[CGT][AGT]GTAGTCCAGAT
3	Athens, GA	7/7/14	Water	NA	6	TGATTGACCCTACGGG[ATCG]GGC[AT]GCAG	GGATTAGATACCC[CGT][AGT]GTAGTCCCTAG
3	Athens, GA	9/23/14	Larvae	*Ae. albopictus*	1	TGATTGACCCTACGGG[ATCG]GGC[AT]GCAG	GGATTAGATACCC[CGT][AGT]GTAGTCAGTCTC
3	Athens, GA	9/23/14	Larvae	*C. quinquefasciatus*	4	TGATTGACCCTACGGG[ATCG]GGC[AT]GCAG	GGATTAGATACCC[CGT][AGT]GTAGTCACTTCGC
3	Athens, GA	9/23/14	Water	NA	18	CTGGATGCCTACGGG[ATCG]GGC[AT]GCAG	GGATTAGATACCC[CGT][AGT]GTAGTCCCACTC
3	Laboratory	9/30/14	Larvae	*Ae. aegypti*	17	CTGGATGCCTACGGG[ATCG]GGC[AT]GCAG	GGATTAGATACCC[CGT][AGT]GTAGTCGACGTGG
3	Laboratory	9/30/14	Larvae	*Ae. albopictus*	15	CTGGATGCCTACGGG[ATCG]GGC[AT]GCAG	GGATTAGATACCC[CGT][AGT]GTAGTCCCTAG
3	Laboratory	9/30/14	Larvae	*C. quinquefasciatus*	12	CTGGATGCCTACGGG[ATCG]GGC[AT]GCAG	GGATTAGATACCC[CGT][AGT]GTAGTCCAGAT
3	Laboratory	9/30/14	Water	*Ae. aegypti*	10	CTGGATGCCTACGGG[ATCG]GGC[AT]GCAG	GGATTAGATACCC[CGT][AGT]GTAGTCGATTGAGA
3	Laboratory	9/30/14	Water	*Ae. albopictus*	9	TGATTGACCCTACGGG[ATCG]GGC[AT]GCAG	GGATTAGATACCC[CGT][AGT]GTAGTCTTCCT
3	Laboratory	9/30/14	Water	*C. quinquefasciatus*	8	TGATTGACCCTACGGG[ATCG]GGC[AT]GCAG	GGATTAGATACCC[CGT][AGT]GTAGTCGACGTGG

Table B.2: One-way ANOVA to compare alpha and beta diversity between technical replicates.

		F	P
Alpha diversity			
	Species richness	0.082	0.921
	Chao1	0.087	0.917
	Shannon's H	0.024	0.976
Beta diversity			
	Unweighted UniFrac distance	1.115	0.328
	Bray-Curtis distance	0.044	0.957

Table B.3: Bacterial isolates used in this study.

Location	**Mosquito species isolate was collected from**	**Colony color and morphology**	**Culture medium**	**Genus**	**GenBank Accession Number**
Laboratory	*Aedes aegypti*	Orange (small, circular, convex, smooth)	Nutrient agar (BHI)	*Chryseobacterium*	KJ192342
Laboratory	*Ae. aegypti*	White (small) + sec	R2A	*Comamonas*	KX260133
Laboratory	*Ae. aegypti*	White-yellow (small, circular, convex, smooth)	Nutrient agar (BHI)	*Sphingobacterium*	KX260132
Laboratory	*Ae. aegypti*	Yellow-green (small, circular, convex, smooth)	Nutrient agar (BHI)	*Aquitalea*	KJ192340
Laboratory	*Ae. aegypti*	Tan-buff (medium, circular, convex, smooth)	Blood agar (TSA base)	*Aeromonas*	KJ192338
Athens, GA	*Ae. albopictus*	White (rough surface, uneven margins)	Nutrient agar (LB)	*Bacillus*	KX260134
Athens, GA	*Ae. albopictus*	Tan-buff (medium, circular, convex, smooth)	Nutrient agar (LB)	*Aeromonas*	KX260135

Table B.4: Prevalence of each OTU (97% sequence identity) in each sample, along with a representative sequence and the taxonomic assignment for each OTU.

*Table available online (PMCID: PMC5118126)

Table B.5: Prevalence of each oligotype in each sample, along with a representative sequence and the taxonomic assignment for each oligotype.

*Table available online (PMCID: PMC5118126)

Table B.6: The dominant OTU for a given phylum (or class) identified from *Aedes aegypti* larvae collected from site 1 in Jacksonville, FL on 7/21/2014 or the *Ae. aegypti* UGAL laboratory culture.

*Table shown on next page

Table B.6 (continued)

		Total number of oligotypes identified in larvae	Two Most Abundant Oligotypes	% Sequence Identity Between: (1) Two Most Abundant Oligotypes (2) All Oligotypes	Representative Sequence	BlastN sequence identity, Accession number, Description, Reference (if available)
SITE 1	**Actinobacteria**					
	Leucobacter sp.	32	GTGCTTAGCACTTA ACCTCCCTACCGGA TCCGTGGAAACTTT GGAAACGTGGAGTA G	(1) 99.8% (2) 88.0%	TACGGGGGGCTGCAGTGGGGAATATTGCACAATGGGCGCAAG CCTGATGCAGCGACGCCGCGTGAGGGATGACGGCCTTCGGGT TGTAAACCTCTTTCACCAGGGACGAAGCGCAAGTGACGGTAC CTGGAGAAGAAGGACCGGCCAACTACGTGCCAGCAGCCGCGG TAATACGTAGGGTCCGAGCGTTGTCCGGAATTACTGGGCGTA AAGAGCTCGTAGGTGGTTTGTCGCGTTGTCCGTGAAAACTCA CAACTCAATTGTGGGCGTGCGGGCGATACGGGCAGACTGGAG TACTGCAGGGGAGACTGGAATTCCTGGTGTAGCGGTGGAATG CGCAGATATCAGGAGGAACACCGGTGGCGAAGGCGGGTCTCT GGGCAGTAACTGACGCTGAGGAGCGAAAGCGTGGGGAGCGAA CAGGATTAGATACCCGTGTAGTC	99%, AB127812, Uncultured bacterial clone from freshwater sediment in Japan, Tamaki *et al.* 2005
			GTGCTTAGCACTTA ACCTCCCTACCGGA TCCGTGGAAACTTT GGAAACGTGCAGTA G		TACGGGGGGCTGCAGTGGGGAATATTGCACAATGGGCGCAAG CCTGATGCAGCGACGCCGCGTGAGGGATGACGGCCTTCGGGT TGTAAACCTCTTTCACCAGGGACGAAGCGCAAGTGACGGTAC CTGGAGAAGAAGGACCGGCCAACTACGTGCCAGCAGCCGCGG TAATACGTAGGGTCCGAGCGTTGTCCGGAATTACTGGGCGTA AAGAGCTCGTAGGTGGTTTGTCGCGTTGTCCGTGAAAACTCA CAACTCAATTGTGGGCGTGCGGGCGATACGGGCAGACTGGAG TACTGCAGGGGAGACTGGAATTCCTGGTGTAGCGGTGGAATG CGCAGATATCAGGAGGAACACCGGTGGCGAAGGCGGGTCTCT GGGCAGTAACTGACGCTGAGGAGCGAAAGCGTGGGGAGCGAA CAGGATTAGATACCCGAGTAGTC	
	Bacteroidetes					
	Dysgonomonas sp.	72	GCTACGCAAGTCTG AAAGCGTACACATT GCATCTGCGAGGAC TCTTCGAGCTCAAC CTAGGGTTTAGCCT AGACCCAATACTCA AGCATCCA	(1) 99.8% (2) 59.5%	TACGGGGGGCTGCAGTAAGGAATATTGGTCAATGGAGGGAAC TCTGAACCAGCCATGCCGCGTGAAGGATGACGGCCCTCTGGG TTGTAAACTTCTTTTGGATGGGGCGAAAAAACGGGATTTATC CTGGACTGACGGTACCATCAGAATAAGCACCGGCTAACTCCG TGCCAGCAGCCGCGGTAATACGGAGGGTGCAAGCGTTATCCG GAATCACTGGGTTTAAAGGGTGCGTAGGCGGTTGTATAAGTC AGGGGTGAAAGGATGCAGCTTAACTGTATAATTGCCTTTGAT ACTGTATGACTTGAATAAGGTTGAGGTTGGCGGAATGTGACA TGTAGCGGTGAAATGCTTAGATATGTCATGGAACACCGATTG CGAAGGCAGCTGACTGGGCCTTTATTGACGCTGAGGCACGAA AGCGTGGGTAGCGAACAGGATTAGATACCCCTGTAGTC	99%, KJ808034, Uncultured bacterial clone from wastewater in China, Wang *et al.* 2014
			GCTACGCAAGTCTG AAAGCGTACACATT GCATCTGCGAGGAC TCTTCGAGCTCAAC CTAGGGTTTAGCCT AGACCCAATACTCA AGCATCGA		TACGGGGGGCTGCAGTAAGGAATATTGGTCAATGGAGGGAAC TCTGAACCAGCCATGCCGCGTGAAGGATGACGGCCCTCTGGG TTGTAAACTTCTTTTGGATGGGGCGAAAAAACGGGATTTATC CTGGACTGACGGTACCATCAGAATAAGCACCGGCTAACTCCG TGCCAGCAGCCGCGGTAATACGGAGGGTGCAAGCGTTATCCG GAATCACTGGGTTTAAAGGGTGCGTAGGCGGTTGTATAAGTC AGGGGTGAAAGGATGCAGCTTAACTGTATAATTGCCTTTGAT ACTGTATGACTTGAATAAGGTTGAGGTTGGCGGAATGTGACA TGTAGCGGTGAAATGCTTAGATATGTCATGGAACACCGATTG CGAAGGCAGCTGACTGGGCCTTTATTGACGCTGAGGCACGAA AGCGTGGGTAGCGAACAGGATTAGATACCCTTGTAGTC	

		Total number of oligotypes identified in larvae	Two Most Abundant Oligotypes	% Sequence Identity Between: (1) Two Most Abundant Oligotypes (2) All Oligotypes	Representative Sequence	BlastN sequence identity, Accession number, Description, Reference (if available)
	Continued from the previous page...					
	Alphaproteobacteria					
	unclassified Alphaproteobacterium	370	AAGCAGTGCGCAAT GCCGCGAAGGCACA GCGCTGTCAATCTT TGGGCTCAACTCGA ACTGCTGGAGGTCT CGTGAGCTCACCCG GTACTGATGCGAAA CT	(1) 99.8% (2) 66.4%	TACGGGAGGCAGCAGTGGGGAATATTGGACAATGGGCGCAAG CCTGATCCAGCAATTCCGCGTGGGTGAAGAAGGTCTTCGGAT TGTAAAGCCCTTTCGGCGGGGACGATGATGACGGTACCCGCA GAAGAAGCCCCGGCTAACTTCGTGCCAGCAGCCGCGGTAATA CGAAGGGGGCTAGCGTTGCTCGGAATGACTGGGCGTAAAGGG CGCGTAGGCGGCCCGCTAAGTCAGGCGTGAAATTCCCGGGCT CAACCCGGGGGCTGCGTTTGAGACTGGCGGGCTTGAGGGCGG GAGAGGCTCGTGGAATTCCCAGTGTAGAGGTGAAATTCGTAG ATATTGGGAAGAACACCGGTGGCGAAGGCGGCGAGCTGGCCC GTTTCTGACGCTGAGGCGCGAAAGCGTGGGGAGCAAACAGGA TTAGATACCCCAGTAGTC	99%, KC574972, Uncultured bacterial clone from a freshwater spring in northern California, US, Suzuki *et al.* 2013
			AAGCAGTGCGCAAT GCCGCGAAGGCACA GCGCTGTCAATCTT TGGGCTCAACTCGA ACTGCTGGAGGTCT CGTGAGCTCACCCG GTACTGATGCGAAA GG		TACGGGAGGCAGCAGTGGGGAATATTGGACAATGGGCGCAAG CCTGATCCAGCAATTCCGCGTGGGTGAAGAAGGTCTTCGGAT TGTAAAGCCCTTTCGGCGGGGACGATGATGACGGTACCCGCA GAAGAAGCCCCGGCTAACTTCGTGCCAGCAGCCGCGGTAATA CGAAGGGGGCTAGCGTTGCTCGGAATGACTGGGCGTAAAGGG CGCGTAGGCGGCCCGCTAAGTCAGGCGTGAAATTCCCGGGCT CAACCCGGGGGCTGCGTTTGAGACTGGCGGGCTTGAGGGCGG GAGAGGCTCGTGGAATTCCCAGTGTAGAGGTGAAATTCGTAG ATATTGGGAAGAACACCGGTGGCGAAGGCGGCGAGCTGGCCC GTTTCTGACGCTGAGGCGCGAAAGCGTGGGGAGCAAACAGGA TTAGATACCCCTGTAGTC	
	Betaproteobacteria					
	C39 sp.	76	GTGACATGTCGCTG ATCGCACAGGCCCT GTGTGGAGCTGGTA GGTTTTATCCTATG AACCTAAAACCGAG TCGCCGATCCAGTA G	(1) 93.7% (2) 84.0%	TACGGGGGGCTGCAGTGGGGAATTTTGGACAATGGGGGAAAC CCTGATCCAGCCATGCCGCGTGAGTGAAGAAGGCCTTCGGGT TGTAAAGCTCTTTCAGACGGAAAGAAAACGAGCAGGTTAATA CCCTGTTTGGATGACGGTACCGTCAGAAGAAGCACCGGCTAA CTACGTGCCAGCAGCCGCGGTAATACGTAGGGTGCGAGCGTT AATCGGAATTACTGGGCGTAAAGCGTGCGCAGGCGGTTGTGT AAGACAGATGTGAAAGCCCCGGGCTTAACCTGGGAACTGCGT TTGTGACTGCACAGCTAGAGTACGGCAGAGGGGGGTGGAATT CCACGTGTAGCAGTGAAATGCGTAGAGATGTGGAGGAACACC GATGGCGAAGGCAGCCCCCTGGGCCTGTACTGACGCTCATGC ACGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTTGTAGT C	99%, FJ608058, Uncultured *Azoarcus* sp. clone from the gut microbiota of *Anopheles stephensi* mosquito larvae collected in India, Rani *et al.* 2009
			GTGACATGTCGCTG ATCGCACAGGCCCT GTGTGGAGCTGGTA GGTTTTATCCTATG AACCTAAAACCGAG TCGCCGATCGAGTA G		TACGGGGGGCTGCAGTGGGGAATTTTGGACAATGGGGGAAAC CCTGATCCAGCCATGCCGCGTGAGTGAAGAAGGCCTTCGGGT TGTAAAGCTCTTTCGGCCGGGAAGAAATCGCACAGGCTAATA CTCTGTGTGGATGACGGTACCGGCATAAGAAGCACCGGCTAA CTACGTGCCAGCAGCCGCGGTAATACGTAGGGTGCGAGCGTT AATCGGAATTACTGGGCGTAAAGCGTGCGCAGGCGGTTAGGT AAGTTTGATGTGAAATCCCCGGGCTCAACCTGGGAACTGCAT TGAAGACTGCCTAACTAGAGTACGGCAGAGGGGGGTGGAATT CCACGTGTAGCAGTGAAATGCGTAGAGATGTGGAGGAACACC GATGGCGAAGGCAGCCCCCTGGGCCGATACTGACGCTCATGC ACGAAAGCGTGGGGAGCAAACAGGATTAGATACCCCGGTAGT C	99%, LC094636, Rhodocyclaceae bacterium identified from the Tokigawa River in Japan

SITE 1

		Total number of oligotypes identified in larvae	Two Most Abundant Oligotypes	% Sequence Identity Between: (1) Two Most Abundant Oligotypes (2) All Oligotypes	Representative Sequence	BlastN sequence identity, Accession number, Description, Reference (if available)
SITE 1	Continued from the previous page...					
	Gammaproteobacteria					
	unclassified Sinobacteraceae	5	GTGCAGTAGCTCTT GTTAATACGGCAGG TTGCTAATGGGTAG	(1) 99.8% (2) 99.6%	TACGGGGGGCTGCAGTGGGGAATATTGCACAATGGGCGCAAG CCTGATGCAGCCATGCCGCGTGTGTGAAGAAGGCCTTCGGGT TGTAAAGCACTTTCAGCGAGGAGGAAGGGGGTGGTGTTAATA TCACCATTCATTGACGTTACTCGCAGAAGAAGCACCGGCTAAC TCCGTGCCAGCAGCCGCGGTAATACGGAGGGTGCAAGCGTTA ATCGGAATTACTGGGCGTAAAGCGCACGCAGGCGGTTTGTTA AGTCAGATGTGAAATCCCCGAGCTTAACTTGGGAACTGCATT TGAAACTGGCAAGCTAGAGTCTTGTAGAGGGGGGTAGAATTC CAGGTGTAGCGGTGAAATGCGTAGAGATCTGGAGGAATACCG GTGGCGAAGGCGGCCCCCTGGACAAAGACTGACGCTCAGGTG CGAAAGCGTGGGGAGCAAACAGGATTAGATACCCGGTAGTC	99%, KT388005, *Serratia* sp. from a water distribution system in Germany
			GTGCAGTAGCTCTT GTTAATACGGCAGG TTGCTAATTAGTAG		TACGGGGGGCTGCAGTGGGGAATATTGCACAATGGGCGCAAG CCTGATGCAGCCATGCCGCGTGTGTGAAGAAGGCCTTCGGGT TGTAAAGCACTTTCAGCGAGGAGGAAGGGGGTGGTGTTAATA TCACCATTCATTGACGTTACTCGCAGAAGAAGCACCGGCTAAC TCCGTGCCAGCAGCCGCGGTAATACGGAGGGTGCAAGCGTTA ATCGGAATTACTGGGCGTAAAGCGCACGCAGGCGGTTTGTTA AGTCAGATGTGAAATCCCCGAGCTTAACTTGGGAACTGCATT TGAAACTGGCAAGCTAGAGTCTTGTAGAGGGGGGTAGAATTC CAGGTGTAGCGGTGAAATGCGTAGAGATCTGGAGGAATACCG GTGGCGAAGGCGGCCCCCTGGACAAAGACTGACGCTCAGGTG CGAAAGCGTGGGGAGCAAACAGGATTAGATACCCGGGTAGTC	
	Deltaproteobacteria					
	Desulfovibrio cuneatus	72	GTGCAGTCGCCTTC AGGAGGAACATCAG TGGCGACTGTCTGG CCTGTGGGTACA	(1) 99.8% (2) 78.4%	TACGGGGGGCTGCAGTAGGGAATATTGCGCAATGGACGAAAG TCTGACGCAGCGACGCCGCGTGAGTGATGAAGGCCTTAGGGT CGTAAAGCTCTGTTGAATGGGAAGAAATAAATGACGGTACCA TTCGAGGAAGGACCGGCTAACTTCGTGCCAGCAGCCGCGGTA ATACGAGGGGTCCTAGCGTTGTTCGGAATCATTGGGCGTAAA GGGTGTGTAGGTGGCTATGTAAGTCAGGTGTGAAAGCCCCGG GCTCAACCCGGGAAGTGCATCTGATACTGCGTAGCTTGAGTA CCGAAGAGGATAGTGGAATTCCAGGTGTAGTGGTGAAATACG TAGATATCTGGAGGAACACCGGTGGCGAAGGCGGCTATCTGG TCGAGTACTGACACTGAGACACGAAAGCATGGGGATCAAACA GGATTAGATACCCGAGTAGTC	99%, KJ756873, Uncultured bacterial clone from wastewater in Tunisia
			GAGCAGTCGCCTTC AGGAGGAACATCAG TGGCGACTGTCTGG CCTGTGGGTACA		TACGGGGGGCAGCAGTAGGGAATATTGCGCAATGGACGAAAG TCTGACGCAGCGACGCCGCGTGAGTGATGAAGGCCTTAGGGT CGTAAAGCTCTGTTGAATGGGAAGAAATAAATGACGGTACCA TTCGAGGAAGGACCGGCTAACTTCGTGCCAGCAGCCGCGGTA ATACGAGGGGTCCTAGCGTTGTTCGGAATCATTGGGCGTAAA GGGTGTGTAGGTGGCTATGTAAGTCAGGTGTGAAAGCCCCGG GCTCAACCCGGGAAGTGCATCTGATACTGCGTAGCTTGAGTA CCGAAGAGGATAGTGGAATTCCAGGTGTAGTGGTGAAATACG TAGATATCTGGAGGAACACCGGTGGCGAAGGCGGCTATCTGG TCGAGTACTGACACTGAGACACGAAAGCATGGGGATCAAACA GGATTAGATACCCGAGTAGTC	

	Total number of oligotypes identified in larvae	Two Most Abundant Oligotypes	% Sequence Identity Between: (1) Two Most Abundant Oligotypes (2) All Oligotypes	Representative Sequence	BlastN sequence identity, Accession number, Description, Reference (if available)
Continued from the previous page...					
Actinobacteria					
unclassified Microbacteriaceae	72	GTGCCTAACACTTA ACTTCCCTACCGAG CTCGTGGAGACTAT GAAATCGTACAGTA G	(1) 99.8% (2) 88.3%	TACGGGGGGCTGCAGTGGGGAATATTGCACAATGGGCGAAAG CCTGATGCAGCAACGCCGCGTGAGGGATGAAGGCCTTCGGGT TGTAAACCTCTTTTAGCAGGGAAGAAGCGAAAGTGACGGTAC CTGCAGAAAAAGCACCGGCTAACTACGTGCCAGCAGCCGCGG TAATACGTAGGGTGCAAGCGTTGTCCGGAATTATTGGGCGTA AAGAGCTCGTAGGCGGTTTGTCACGTCTGCTGTGAAAATCCG AGGCTCAACCTCGGACCTGCAGTGGGTACGGGCAAGCTAGAG TGCGGTAGGGGAGATGGGAATTCCTGGTGTAGCGGTAAAATG CGCAGATATCAGGAGGAACACCAATGGCGAAGGCACATCTCT GGGCCGTAACTGACGCTGAGGAGCGAAAGCGTGGGGAGCAAA CAGGATTAGATACCCGAGTAGTC	99%, GU369375, Uncultured bacterial clone from the microbiota of a zebra mussel (*Dreissena polymorpha*) in the US Great Lakes
		GTGCCTAACACTTA ACTTCCCTACCGAG CTCGTGGAGACTAT GAAATCGTACGGTA G		TACGGGGGGCTGCAGTGGGGAATATTGCACAATGGGCGAAAG CCTGATGCAGCAACGCCGCGTGAGGGATGAAGGCCTTCGGGT TGTAAACCTCTTTTAGCAGGGAAGAAGCGAAAGTGACGGTAC CTGCAGAAAAAGCACCGGCTAACTACGTGCCAGCAGCCGCGG TAATACGTAGGGTGCAAGCGTTGTCCGGAATTATTGGGCGTA AAGAGCTCGTAGGCGGTTTGTCACGTCTGCTGTGAAAATCCG AGGCTCAACCTCGGACCTGCAGTGGGTACGGGCAAGCTAGAG TGCGGTAGGGGAGATGGGAATTCCTGGTGTAGCGGTAAAATG CGCAGATATCAGGAGGAACACCAATGGCGAAGGCACATCTCT GGGCCGTAACTGACGCTGAGGAGCGAAAGCGTGGGGAGCAAA CAGGATTAGATACCCCAGTAGTC	
Bacteroidetes					
Chryseobacterium sp.	35	ACTGTGAGAGCCTG CCCTTTCTAAAGCT GAAGCTGCAAGATT GTTTCATACTTAAC TAAACAGGAGGTAA TTTACCAATGTCGA TGGAGCCG	(1) 79.3% (2) 71.4%	TACGGGAGGCTGCAGTGAGGAATATTGGTCAATGGGCGAAAG CCTGAACCAGCCAAATCGCGTGAAGGAAGACAGCCCTACGGG TTTTAAACTTCTTTTGTAAGGGAATAAAAAAAGTTACGCGTA ACTTATTGCATGTACCTTACGAATAAGCATCGGCTAACTCCGT GCCAGCAGCCGCGGTAATACGGAGGATGCGAGCGTTATCCGG ATTTATTGGGTTTAAAGGGTGCGTAGGCGGAGACCTAAGTCA GCGGTGAAATCTCGAGGCTCAACCTCGAACCTGCCGTTGAAA CTGAGTCCCTTGAGTATAGATGAAGTAGGCGGAACTCGTTGT GTAGCGGTGAAATGCATAGATATAACGAAGAACCCCAATTGC GTAGGCAGCTTACTAAACTATCACTGACGCTGAGGCACGAAA GCGTGGGTATCAAACAGGATTAGATACCCTAGTAGTC	99%, EF176899, Uncultured Bacteroidales bacterium clone from the hindgut microbiota of giant crane fly (*Tipula abdominalis*) larvae collected from a stream in Michigan, US, Cook *et al.* 2007
		GCTGTGAGAGCCTG CCCTTTCTAAAGCT GAAGCTGCAAGATT GTTTCATACTTAAC TAAACAGGAGGTAA TTTACCAATGTCGA TGGAGCCT		TACGGGGGGCTGCAGTGAGGAATATTGGTCAATGGGCGCAAG CCTGAACCAGCCATGCCGCGTGCAGGAAGACGGTCCTATGGA TTGTAAACTGCTTTTATACAGGAAGAAACAACTCTACGTGTA GAGTCTTGACGGTACTGTAGGAATAAGGATCGGCTAACTCCG TGCCAGCAGCCGCGGTAATACGGAGGATCCAAGCGTTATCCG GAATCATTGGGTTTAAAGGGTCCGTAGGCGGTCTTATAAGTC AGTGGTGAAAGCCCATCGCTCAACGATGGAACTGCCATTGAT ACTGTAAGACTTGAATGCTTAGGAAGTAACTAGAATATGTAG TGTAGCGGTGAAATGCTTAGATATTACATGGAATACCAATTG CGAAGGCAGGTTACTACTAAGTGATTGACGCTGATGGACGAA AGCGTGGGGAGCGAACAGGATTAGATACCCTTGTAGTC	99%, GU305841, Uncultured bacterial clone from an oligotrophic lake in China

LAB

	Total number of oligotypes identified in larvae	Two Most Abundant Oligotypes	% Sequence Identity Between: (1) Two Most Abundant Oligotypes (2) All Oligotypes	Representative Sequence	BlastN sequence identity, Accession number, Description, Reference (if available)
Continued from the previous page...					
Firmicutes					
Clostridium sp.	110	GGCTGGGAAACCCT GTCCCGTTCCGACC GGGCGGAGACGATC TACGTCGCGACTTT ACGTTTAGCCCAGT AG----	(1) 99.8% (2) 79.9%	TACGGGGGGCTGCAGTGGGGAATATTGCACAATGGGGGAAAC CCTGATGCAGCAACGCCGCGTGAGTGATGACGGTCTTCGGAT TGTAAAGCTCTGTCTTCAGGGACGATAATGACGGTACCTGAG GAGGAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATA CGTAGGTGGCAAGCGTTGTCCGGATTTACTGGGCGTAAAGGG AGCGTAGGTGGATATTTAAGTGGGATGTGAAATACTCGGGCT TAACCTGGGTGCTGCATTCCAAACTGGATATCTAGAGTGCAG GAGAGGAAAGTAGAATTCCTAGTGTAGCGGTGAAATGCGTAG AGATTAGGAAGAATACCAGTGGCGAAGGCGACTTTCTGGACT GTAACTGACACTGAGGCTCGAAAGCGTGGGGAGCAAACAGGA TTAGATACCCCAGTAGTC	99%, JQ379125, Uncultured bacterial clone from soil in the US, Dunbar *et al.* 2012
		GGCTGGGAAACCCT GTCCCGTTCCGACC GGGCGGAGACGATC TACGTCGCGACTTT ACGTTTAGCCCTGT AG----		TACGGGGGGCTGCAGTGGGGAATATTGCACAATGGGGGAAAC CCTGATGCAGCAACGCCGCGTGAGTGATGACGGTCTTCGGAT TGTAAAGCTCTGTCTTCAGGGACGATAATGACGGTACCTGAG GAGGAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATA CGTAGGTGGCAAGCGTTGTCCGGATTTACTGGGCGTAAAGGG AGCGTAGGTGGATATTTAAGTGGGATGTGAAATACTCGGGCT TAACCTGGGTGCTGCATTCCAAACTGGATATCTAGAGTGCAG GAGAGGAAAGTAGAATTCCTAGTGTAGCGGTGAAATGCGTAG AGATTAGGAAGAATACCAGTGGCGAAGGCGACTTTCTGGACT GTAACTGACACTGAGGCTCGAAAGCGTGGGGAGCAAACAGGA TTAGATACCCGAGTAGTC	
Betaproteobacteria					
unclassified Comamonadaceae	145	GTCCGCGCGTTCGG ATCTTCTGGGCCCG CAGGAGCCGAACTT GTGGACGATACCAT TGGCACGTTGGGCA GAAAGCTGCCCAGT A	(1) 99.6% (2) 92.0%	TACGGGGGGCTGCAGTGGGGAATTTTGGACAATGGGCGCAAG CCTGATCCAGCCATTCCGCGTGCAGGATGAAGGCCCTCGGGT TGTAAACTGCTTTTGTACGGAACGAAACGGTCCTGGTTAATA CCTGGGGCTAATGACGGTACCGTAAGAATAAGCACCGGCTAA CTACGTGCCAGCAGCCGCGGTAATACGTAGGGTGCAAGCGTT AATCGGAATTACTGGGCGTAAAGCGTGCGCAGGCGGTGATGT AAGACGGATGTGAAATCCCCGGGCTCAACCTGGGAACTGCAT TCGTGACTGCATCGCTGGAGTGCGGCAGAGGGGGATGGAATT CCGCGTGTAGCAGTGAAATGCGTAGATATGCGGAGGAACACC GATGGCGAAGGCAATCCCCTGGGCCTGCACTGACGCTCATGC ACGAAAGCGTGGGGAGCAAACAGGATTAGATACCCGAGTAGT C	99%, KX508966, Uncultured bacterial clone from rainwater collected in China
		GTCCGCGCGTTCGG ATCTTCTGGGCCCG CAGGAGCCGAACTT GTGGACGATACCAT TGGCACGTTGGGCA GAAAGCTGCCTAGT A		TACGGGGGGCTGCAGTGGGGAATTTTGGACAATGGGCGCAAG CCTGATCCAGCCATTCCGCGTGCAGGATGAAGGCCCTCGGGT TGTAAACTGCTTTTGTACGGAACGAAACGGTCCTGGTTAATA CCTGGGGCTAATGACGGTACCGTAAGAATAAGCACCGGCTAA CTACGTGCCAGCAGCCGCGGTAATACGTAGGGTGCAAGCGTT AATCGGAATTACTGGGCGTAAAGCGTGCGCAGGCGGTGATGT AAGACGGATGTGAAATCCCCGGGCTCAACCTGGGAACTGCAT TCGTGACTGCATCGCTGGAGTGCGGCAGAGGGGGATGGAATT CCGCGTGTAGCAGTGAAATGCGTAGATATGCGGAGGAACACC GATGGCGAAGGCAATCCCCTGGGCCTGCACTGACGCTCATGC ACGAAAGCGTGGGGAGCAAACAGGATTAGATACCCCTGTAGT C	

LAB

		Total number of oligotypes identified in larvae	Two Most Abundant Oligotypes	% Sequence Identity Between: (1) Two Most Abundant Oligotypes (2) All Oligotypes	Representative Sequence	BlastN sequence identity, Accession number, Description, Reference (if available)
LAB	Continued from the previous page...					
	Verrucomicrobia					
	Luteolibacter sp.	42	GTGCAGTCGAGAAT AATTCACATCGGTG TTACCCCAGTAG	(1) 99.6% (2) 99.1%	TACGGGAGGCAGCAGTCGAGAATAATTCACAATGGGGGAAAC CCTGATGGTGCAACGCCGCGTGGAGGATGAAGGTCTTCGGAT TGTAAACTCCTGTCATCTGGGAGTAAGACCTGGCTGTGAATA GCAGACAGGGTTGATAGTACCAGAAGAGGAAGGGACGGCTAA CTTCGTGCCAGCAGCCGCGGTAATACGAAGGTCCCAAGCGTT GTTCGGAATCACTGGGCGTAAAGGGAGCGTAGGCGGCGTGGT AAGTCAGATGTGAAATCCCGGGGCTCAACCCCGGAACTGCAT CCGATACTGCCATGCTTGAGGGTTGGAGAGGTAGCTGGAATT CTCGGTGTAGCAGTGAAATGCGTGGATATCGAGAGGAACACT CGTGGCGAAGGCGAGCTACTGGACAACATCTGACGCTGAGGC TCGAAGGCTAGGGGAGCGAAAGGGATTAGATACCCCAGTAGT C	99%, AB240273, Uncultured bacterial clone from laboratory-based common reed (*Phragmites australis*) bed reactor in Japan
			GTGCAGTCGAGAAT AATTCACATCGGTG TTACCCGTGTAG		TACGGGAGGCAGCAGTCGAGAATAATTCACAATGGGGGAAAC CCTGATGGTGCAACGCCGCGTGGAGGATGAAGGTCTTCGGAT TGTAAACTCCTGTCATCTGGGAGTAAGACCTGGCTGTGAATA GCAGACAGGGTTGATAGTACCAGAAGAGGAAGGGACGGCTAA CTTCGTGCCAGCAGCCGCGGTAATACGAAGGTCCCAAGCGTT GTTCGGAATCACTGGGCGTAAAGGGAGCGTAGGCGGCGTGGT AAGTCAGATGTGAAATCCCGGGGCTCAACCCCGGAACTGCAT CCGATACTGCCATGCTTGAGGGTTGGAGAGGTAGCTGGAATT CTCGGTGTAGCAGTGAAATGCGTGGATATCGAGAGGAACACT CGTGGCGAAGGCGAGCTACTGGACAACATCTGACGCTGAGGC TCGAAGGCTAGGGGAGCGAAAGGGATTAGATACCCTTGTAGT C	

Table B.7: Minimal inhibitory concentrations (μg/ml) for select antibiotics against individual members of the gut microbiota isolated from UGAL *Aedes aegypti* or field-collected *Ae. albopictus* larvae.

*Table shown on next page

Table B.7 (continued)

Bacterial isolate	Ampicillin	Kanamycin	Streptomycin	Chloramphenicol	Penicillin	Tetracycline	Gentamicin
Ae. aegypti							
Chryseobacterium sp.	>200	>200	>200	200	>200	200	>200
Comamonas sp.	>200	200	200	200	>200	20	20
Sphingobacterium sp.	>200	>200	>200	20	>200	20	200
Aquitalea sp.	>200	20	200	20	200	2	20
Aeromonas sp.	>200	20	20	200	200	20	20
Ae. albopictus							
Bacillus sp.	>200	200	200	20	200	2	20
Aeromonas sp.	>200	200	200	2	200	2	20

B.3 Supplementary Figures

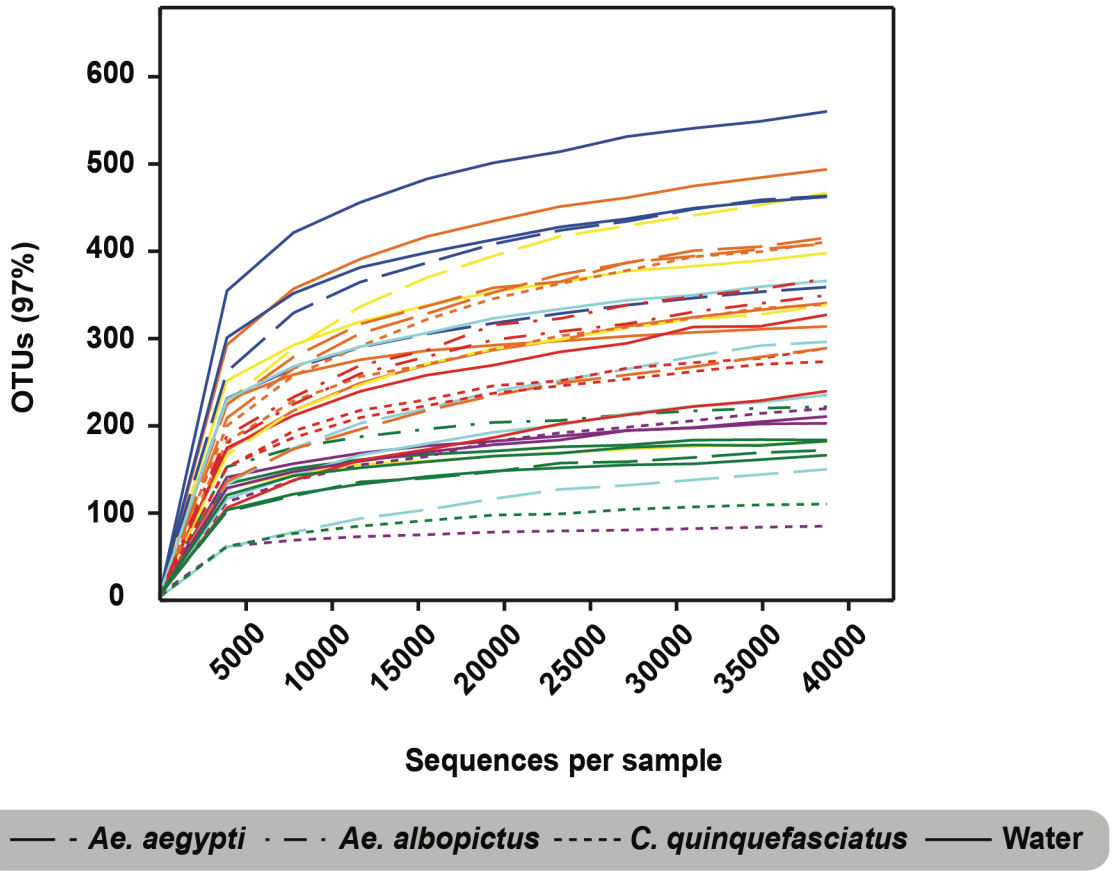
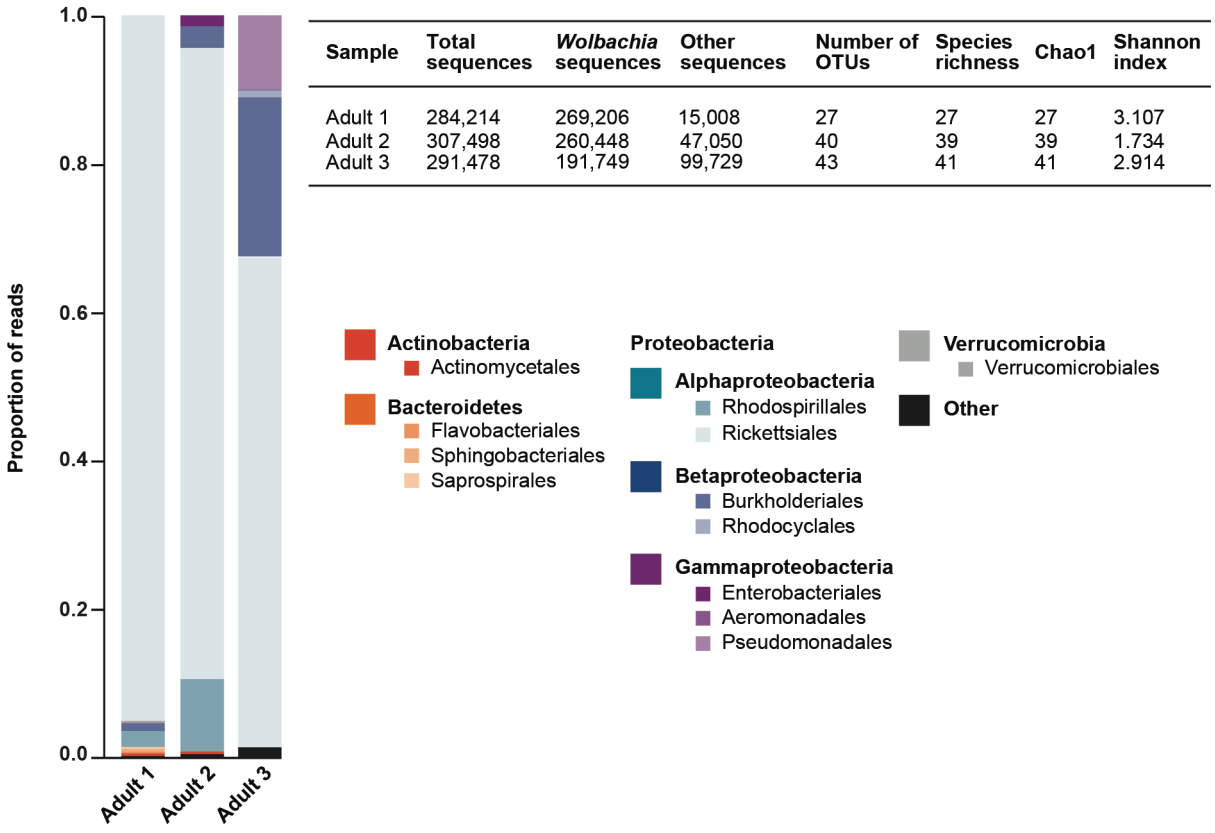


Figure B.1: Rarefaction data from Illumina sequencing experiments. Reads from each water and mosquito library were sampled starting at 100 sequences per step and increased in increments of 50 until the total number of reads per sample was reached. Each step was sampled 10 times. Each line represents the smoothed mean across each increment for OTUs clustered at a sequence similarity threshold of 97%. Lines for each larval and water sample across all field and laboratory sites and sampling dates are shown. Each site and sampling date is shown with a different color while each species or water sample is indicated by different line fonts as shown in the legend.



Sample	Total sequences	*Wolbachia* sequences	Other sequences	Number of OTUs	Species richness	Chao1	Shannon index
Adult 1	284,214	269,206	15,008	27	27	27	3.107
Adult 2	307,498	260,448	47,050	40	39	39	1.734
Adult 3	291,478	191,749	99,729	43	41	41	2.914

Figure B.2: Sequence data for the 16S rRNA libraries from three adult *Aedes aegypti* that emerged from site 1 in Jacksonville, FL. The upper right of the figure presents sequencing statistics. Bars to left indicate the proportion of sequencing reads assigned to a given bacterial order. Only categories >2% are presented.

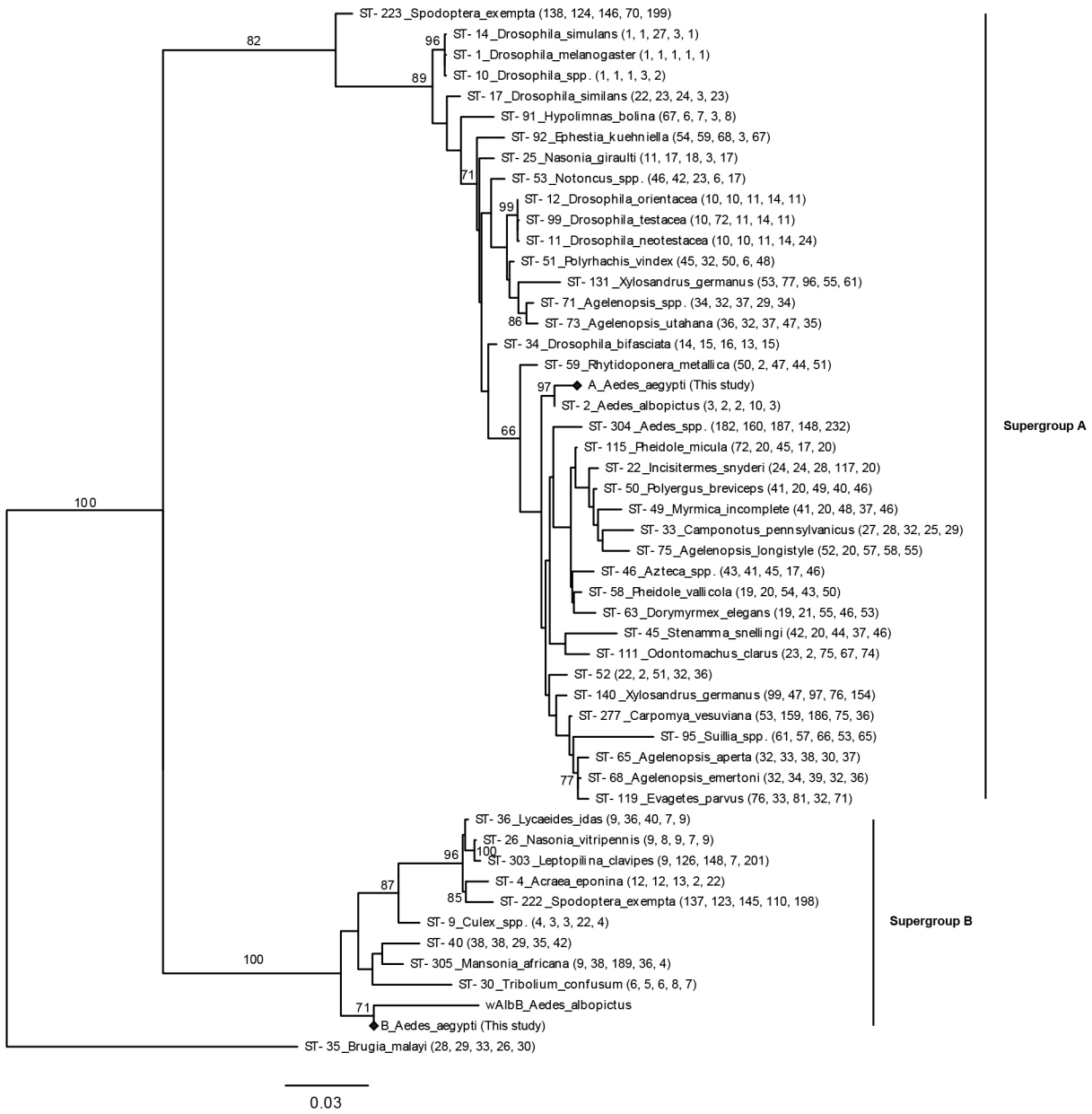


Figure B.3: Maximum-likelihood phylogenetic tree for concatenated MLST genes of *Wolbachia* isolates. The solid diamonds indicate the 2 strains identified from *Aedes aegypti* in this study. Where known, the names of the host species are given. The scale bar represents a 3% estimated difference in nucleotide sequence. Numbers given at each node correspond to the percentage bootstrap values (for 1,000 repetitions). Replicate numbers of <60% were not included in the figure. *Brugia malayi Wolbachia* ST-35 is used as an outgroup. Where available, the allelic profile for the assigned sequence type (ST) is provided in parentheses (*gatB*, *coxA*, *hcpA*, *fbpA*, *ftsZ*).

APPENDIX C

SUPPORTING INFORMATION FOR CHAPTER 4

C.1 Supplementary Tables

Table C.1: Keio mutants defective in stimulating larval growth and molting.

*Table shown on next page

Table C.1 (continued)

Defective subclass	JW#	Gene name	Gene description	Functional category	Recovered from water?[a]	Recovered from larvae?[b]	Conditional on inoculum density?[c]
growth	JW0794	glnQ	L-glutamine ABC transporter, ATP binding subunit	amino acid biosynthesis/transport	N	N	Y
growth	JW3706	pstS	phosphate ABC transporter, periplasmic binding protein	cofactor synthesis/transport	N	N	Y
growth	JW3730	rbsB	ribose ABC transporter, putative periplasmic binding protein	cofactor synthesis/transport	N	N	Y
growth	JW0450	ybaJ	protein that modulates Hha toxicity	stress response	N	N	Y
growth	JW1152	ymgA	protein involved in biofilm formation	stress response	N	N	NT
growth	JW0656	ybeY	endoribonuclease	stress response	N	N	Y
growth	JW0695	ybfL	predicted transposase; receptor protein	stress response	N	N	Y
growth	JW4366	yjtD	predicted rRNA methyltransferase	stress response	N	N	Y
growth	JW3822	fadB	fatty acid oxidation complex, α component	fatty acid metabolism	N	N	Y
growth	JW2880	serA	2-oxoglutarate reductase	amino acid biosynthesis/transport	N	N	Y
growth	JW0890	serC	3-phosphoserine aminotransferase	amino acid biosynthesis/transport	N	N	NT
growth	JW0854	ltaE	L-threonine aldolase	amino acid biosynthesis/transport	N	N	Y
growth	JW3975	aceA	isocitrate lyase	central metabolism	N	N	NT
growth	JW1839	eda	multifunctional aldolase/decarboxylase	central metabolism	N	N	Y
growth	JW1838	purT	phosphoribosylglycinamide formyltransferase 2	central metabolism	N	N	Y
growth	JW2471	hyfF	hydrogenase 4, component F	central metabolism	N	N	NT
growth	JW2128	dusC	tRNA-dihydrouridine synthase C	central metabolism	N	N	Y
growth	JW2466	hyfA	hydrogenase 4, component A	central metabolism	N	N	NT
growth	JW1375	ldhA	D-lactate dehydrogenase	central metabolism	N	N	Y
growth	JW1821	yebR	methionine-(*R*)-sulfoxide reductase	stress response	N	N	Y
growth	JW2133	yeiT	NADH-dependent dihydropyrimidine dehydrogenase subunit	central metabolism	N	N	Y
growth	JW2670	ygaD	ribonucleoside-diphosphate reductase 2, β subunit dimer	central metabolism	N	N	Y
growth	JW2970	yghZ	L-glyceraldehyde 3-phosphate reductase	stress response	N	N	Y
growth	JW3349	rpe	ribulose-5-phosphate 3-epimerase	central metabolism	N	N	Y
growth	JW1841	zwf	glucose-6-phosphate dehydrogenase	central metabolism	N	N	Y
growth	JW1483	ddpX	D-Ala-D-Ala dipeptidase	peptidoglycan biosynthesis/recycling	N	N	Y
growth	JW3175	mtgA	biosynthetic peptidoglycan transglycosylase	peptidoglycan biosynthesis/recycling	N	N	Y
growth	JW0427	clpP	ClpP serine protease	stress response	N	N	Y
growth	JW0865	clpS	specificity factor for ClpA-ClpP chaperone-protease complex	stress response	N	N	Y
growth	JW0885	pflA	pyruvate formate-lyase activating enzyme	central metabolism	N	N	Y
growth	JW3580	lldD	L-lactate dehydrogenase	central metabolism	N	N	Y
growth	JW1666	pykF	pyruvate kinase I	central metabolism	N	N	Y
growth	JW0807	ybiW	predicted pyruvate formate lyase	central metabolism	N	N	Y
growth	JW2213	atoS	AtoS sensory histidine kinase	regulatory process	N	N	Y
growth	JW3905	cytR	CytR DNA-binding transcriptional repressor	regulatory process	N	N	NT
growth	JW4085	dcuR	DcuR transcriptional activator	regulatory process	N	N	Y
growth	JW3195	nanR	NanR DNA-binding transcriptional dual regulator	regulatory process	N	N	Y
growth	JW0019	nhaR	NhaR transcriptional activator	regulatory process	N	N	Y
growth	JW4200	treR	TreR DNA-binding transcriptional repressor	regulatory process	N	N	Y
growth	JW0198	yafC	putative LysR family transcriptional regulator	regulatory process	N	N	Y
growth	JW0276	yagP	predicted transcriptional regulator LYSR-type	regulatory process	N	N	Y
growth	JW0310	yahD	predicted transcriptional regulator with ankyrin domain	regulatory process	N	N	Y

[a]Y: viable colonies recovered after plating 100 µl of water from larval cultures 48 h post-inoculation; N: no viable colonies recovered

[b]Y: viable colonies recovered after plating homogenates of larvae 48 h post-inoculation; N: no viable colonies recovered

[c]Y: supplementation of larval cultures with 10^5 colony-forming units (CFUs) resulted in >80% of larvae molting to the second instar within 48 h; NT: not tested

Defective subclass	JW#	Gene name	Gene description	Functional category	Recovered from water?[a]	Recovered from larvae?[b]	Conditional on inoculum density?[c]
Continued from the previous page...							
growth	JW3087	tdcC	serine / threonine:H^+ symporter TdcC	amino acid biosynthesis/transport	N	N	Y
growth	JW3124	yhbQ	DNA nuclease / DNA damage response protein	stress response	N	N	NT
growth	JW4233	yjgW	KpLE2 phage-like element; predicted protein	stress response	N	N	NT
growth	JW0455	ybaM	small protein involved in the cell envelope stress response	stress response	N	N	Y
growth	JW5058	yajQ	nucleotide binding protein	stress response	N	N	Y
growth	JW1252	trpA	tryptophan synthase, α subunit	amino acid biosynthesis/transport	N	N	Y
colonization	JW5092	gltI	glutamate / aspartate ABC transporter, periplasmic binding protein	amino acid biosynthesis/transport	Y	N	Y
colonization	JW0649	gltJ	glutamate / aspartate ABC transporter, membrane subunit	amino acid biosynthesis/transport	Y	N	Y
colonization	JW3419	livF	branched chain amino acid / phenylalanine ABC transporter, ATP binding	amino acid biosynthesis/transport	Y	N	Y
colonization	JW0748	modC	molybdate ABC transporter, ATP binding subunit	cofactor synthesis/transport	Y	N	Y
colonization	JW3444	nikD	nickel ABC transporter, ATP binding subunit	cofactor synthesis/transport	Y	N	Y
colonization	JW4067	phnC	phosphonate ABC transporter, ATP binding subunit	cofactor synthesis/transport	Y	N	Y
colonization	JW4066	phnD	phosphonate ABC transporter, periplasmic binding protein	cofactor synthesis/transport	Y	N	Y
colonization	JW1112	potA	putrescine / spermidine ABC transporter, ATP binding subunit	cofactor synthesis/transport	Y	N	Y
colonization	JW2653	proW	glycine betaine ABC transporter, membrane subunit	amino acid biosynthesis/transport	Y	N	Y
colonization	JW3728	rbsA	ribose ABC transporter, putative ATP binding subunit	cofactor synthesis/transport	Y	N	Y
colonization	JW0435	ybaE	putative transport protein, periplasmic-binding component of ABC superfamily	amino acid biosynthesis/transport	Y	N	Y
colonization	JW2118	yehY	glycine betaine ABC transporter, putative membrane subunit	amino acid biosynthesis/transport	Y	N	Y
colonization	JW3712	atpA	ATP synthase F_1 complex, alpha subunit	other	Y	N	Y
colonization	JW3711	atpG	ATP synthase F_1 complex, gamma subunit	other	Y	N	Y
colonization	JW0757	bioA	adenosylmethionine-8-amino-7-oxononanoate aminotransferase	cofactor synthesis/transport	Y	N	Y
colonization	JW0758	bioB	biotin synthase	cofactor synthesis/transport	Y	N	Y
colonization	JW5130	yccU	predicted CoA-binding protein with NAD(P)-binding Rossmann-fold domain	central metabolism	Y	N	Y
colonization	JW2884	yliK	methylmalonyl-CoA mutase	central metabolism	Y	N	Y
colonization	JW2216	atoA	acetyl-CoA:acetoacetyl-CoA transferase, beta subunit	fatty acid metabolism	Y	N	Y
colonization	JW2215	atoD	acetyl-CoA:acetoacetyl-CoA transferase, alpha subunit	fatty acid metabolism	Y	N	Y
colonization	JW2218	atoB	acetyl-CoA acetyltransferase	fatty acid metabolism	Y	N	Y
colonization	JW2217	atoE	predicted short chain fatty acid transporter	fatty acid metabolism	Y	N	Y
colonization	JW5020	fadE	acyl-CoA dehydrogenase	fatty acid metabolism	Y	N	Y
colonization	JW5156	plsX	fatty acid/phospholipid synthesis protein	fatty acid metabolism	Y	N	Y
colonization	JW3841	glnA	glutamine synthetase	amino acid biosynthesis/transport	Y	N	Y
colonization	JW2872	gcvH	glycine cleavage system H protein	amino acid biosynthesis/transport	Y	N	Y
colonization	JW2871	gcvP	glycine decarboxylase	amino acid biosynthesis/transport	Y	N	Y
colonization	JW2873	gcvT	aminomethyltransferase	amino acid biosynthesis/transport	Y	N	Y
colonization	JW2535	glyA	serine hydroxymethyltransferase	amino acid biosynthesis/transport	Y	N	Y
colonization	JW1803	sdaA	L-serine deaminase I	amino acid biosynthesis/transport	Y	N	Y
colonization	JW0002	thrB	homoserine kinase	amino acid biosynthesis/transport	Y	N	Y
colonization	JW4358	ytjC	predicted phosphatase	central metabolism	Y	N	Y
colonization	JW3863	fdoI	formate dehydrogenase-O, γ subunit	central metabolism	Y	N	Y
colonization	JW2946	glcD	glycolate oxidase, predicted FAD-linked subunit	central metabolism	Y	N	Y
colonization	JW5486	glcF	glycolate oxidase, predicted iron-sulfur subunit	central metabolism	Y	N	Y
colonization	JW1220	purU	formyltetrahydrofolate deformylase	central metabolism	Y	N	Y
colonization	JW1495	ydeP	acid resistance protein	stress response	Y	N	Y
colonization	JW2303	hisP	lysine/arginine/ornithine/histidine ABC transporter, ATP binding	amino acid biosynthesis/transport	Y	N	Y

[a]Y: viable colonies recovered after plating 100 µl of water from larval cultures 48 h post-inoculation; N: no viable colonies recovered

[b]Y: viable colonies recovered after plating homogenates of larvae 48 h post-inoculation; N: no viable colonies recovered

[c]Y: supplementation of larval cultures with 10^5 CFUs resulted in >80% of larvae molting to the second instar within 48 h; NT: not tested

Defective subclass	JW#	Gene name	Gene description	Functional category	Recovered from water?[a]	Recovered from larvae?[b]	Conditional on inoculum density?[c]
Continued from the previous page...							
colonization	JW3606	rfaG	lipopolysaccharide glucosyltransferase I	LPS biosynthesis/modification	Y	N	Y
colonization	JW0212	lpcA	D-sedoheptulose 7-phosphate isomerase	LPS biosynthesis/modification	Y	N	Y
colonization	JW3985	pgi	phosphoglucose isomerase	LPS biosynthesis/modification	Y	N	Y
colonization	JW3595	rfaF	ADP-heptose:LPS heptosyltransferase II	LPS biosynthesis/modification	Y	N	Y
colonization	JW3601	rfaJ	UDP-glucose:(glucosyl)LPS α-1,2-glucosyltransferase	LPS biosynthesis/modification	Y	N	Y
colonization	JW3597	rfaL	O-antigen ligase	LPS biosynthesis/modification	Y	N	Y
colonization	JW3979	metH	cobalamin-dependent methionine synthase	amino acid biosynthesis/transport	Y	N	Y
colonization	JW3459	yhiN	predicted oxidoreductase with FAD/NAD(P)-binding domain	central metabolism	Y	N	Y
colonization	JW3853	yihU	3-sulfolactaldehyde reductase	stress response	Y	N	Y
colonization	JW4346	deoB	phosphopentomutase	central metabolism	Y	N	Y
colonization	JW4344	deoC	deoxyribose-phosphate aldolase	central metabolism	Y	N	Y
colonization	JW0007	talB	transaldolase B	central metabolism	Y	N	Y
colonization	JW0106	ampD	*N*-acetyl-anhydromuramyl-L-alanine amidase	peptidoglycan biosynthesis/recycling	Y	N	Y
colonization	JW0823	dacC	D-alanyl-D-alanine carboxypeptidase	peptidoglycan biosynthesis/recycling	Y	N	Y
colonization	JW5329	dacD	DD-carboxypeptidase, penicillin-binding protein 6b	peptidoglycan biosynthesis/recycling	Y	N	Y
colonization	JW5646	envC	EnvC divisome associated factor, activator of peptidoglycan hydrolases	peptidoglycan biosynthesis/recycling	Y	N	Y
colonization	JW0210	ivy	periplasmic chaperone, inhibitor of vertebrate C-type lysozyme	peptidoglycan biosynthesis/recycling	Y	N	Y
colonization	JW1181	ldcA	L,D-carboxypeptidase A	peptidoglycan biosynthesis/recycling	Y	N	Y
colonization	JW2325	mepA	murein DD-endopeptidase	peptidoglycan biosynthesis/recycling	Y	N	Y
colonization	JW1093	nagZ	β-N-acetylhexosaminidase	peptidoglycan biosynthesis/recycling	Y	N	Y
colonization	JW0851	ybjR	anhydro-N-acetylmuramoyl-L-alanine amidase	peptidoglycan biosynthesis/recycling	Y	N	Y
colonization	JW0908	ycbB	L,D-transpeptidase	peptidoglycan biosynthesis/recycling	Y	N	Y
colonization	JW5304	yebA	murein DD-endopeptidase	peptidoglycan biosynthesis/recycling	Y	N	Y
colonization	JW0111	aceF	pyruvate dehydrogenase, E2 subunit	central metabolism	Y	N	Y
colonization	JW0110	aceE	pyruvate dehydrogenase	central metabolism	Y	N	Y
colonization	JW4030	acs	acetyl-CoA synthetase (AMP-forming)	central metabolism	Y	N	Y
colonization	JW1412	aldA	aldehyde dehydrogenase A, NAD-linked	central metabolism	Y	N	Y
colonization	JW1643	gloA	glyoxalase I	central metabolism	Y	N	Y
colonization	JW0342	mhpF	acetaldehyde dehydrogenase (acylating)	central metabolism	Y	N	Y
colonization	JW2198	mqo	malate:quinone oxidoreductase	central metabolism	Y	N	Y
colonization	JW3366	pck	phosphoenolpyruvate carboxykinase (ATP)	central metabolism	Y	N	Y
colonization	JW0886	pflB	pyruvate formate-lyase (inactive)	central metabolism	Y	N	Y
colonization	JW2294	pta	phosphate acetyltransferase / phosphate propionyltransferase	central metabolism	Y	N	Y
colonization	JW0390	phoR	PhoR sensory histidine kinase	regulatory process	Y	N	Y
colonization	JW0710	gltA	citrate synthase	central metabolism	Y	N	Y
colonization	JW0112	lpd	lipoamide dehydrogenase	central metabolism	Y	N	Y
colonization	JW1253	trpB	tryptophan synthase, β subunit dimer	amino acid biosynthesis/transport	Y	N	Y
colonization	JW2317	pdxB	erythronate-4-phosphate dehydrogenase	cofactor synthesis/transport	Y	N	Y
colonization	JW2548	pdxJ	pyridoxine 5'-phosphate synthase	cofactor synthesis/transport	Y	N	Y
colonization	JW2894	epd	erythrose 4-phosphate dehydrogenase	cofactor synthesis/transport	Y	N	Y
colonization	JW1628	pdxY	pyridoxal kinase 2	cofactor synthesis/transport	Y	N	Y
rescue	JW4364	arcA	ArcA transcriptional dual regulator	regulatory process/respiration	Y	Y	-
rescue	JW0723	cydB	cytochrome *bd*-I terminal oxidase subunit II	respiration	Y	Y	-
rescue	JW0870	cydD	ATP-binding/permease protein	respiration	Y	Y	-

[a]Y: viable colonies recovered after plating 100 µl of water from larval cultures 48 h post-inoculation; N: no viable colonies recovered

[b]Y: viable colonies recovered after plating homogenates of larvae 48 h post-inoculation; N: no viable colonies recovered

[c]Y: supplementation of larval cultures with 10^5 CFUs resulted in >80% of larvae molting to the second instar within 48 h; NT: not tested

Defective subclass	JW#	Gene name	Gene description	Functional category	Recovered from water?[a]	Recovered from larvae?[b]	Conditional on inoculum density?[c]
Continued from the previous page...							
rescue	JW1328	fnr	FNR DNA-binding transcriptional dual regulator	regulatory process/respiration	Y	Y	-

[a]Y: viable colonies recovered after plating 100 µl of water from larval cultures 48 h post-inoculation; N: no viable colonies recovered

[b]Y: viable colonies recovered after plating homogenates of larvae 48 h post-inoculation; N: no viable colonies recovered

[c]Y: supplementation of larval cultures with 10^5 CFUs resulted in >80% of larvae molting to the second instar within 48 h; NT: not tested

Table C.2: Primers designed against the *Escherichia coli* BW25113 genome (NCBI Accession No. CP009273) and used to contruct the Δ*cydB*-Δ*cydD*::*kan* and Δ*narZ*-Δ*narG*::*kan* double mutants.*

Name	Sequence (5'-3')	Target gene	Reference
k2	CGGTGCCCTGAATGAACTGC	*kan*	Datsenko & Wanner 2000
kt	CGGCCACAGTCGATGAATCC	*kan*	Datsenko & Wanner 2000
cydB-U	GCAGGCGATCTCATCTTCTC	*cydA*	This study
cydB-D	AAGAAGCGTTCCCAGAATCC	*cydX*	This study
cydD-U	CACCACGGAAGTGGAAAACT	*trxB*	This study
cydD-D	CGCTGATGGTCTGTCGTAGA	*cydC*	This study
narG-U	GCTTTGCAATGCTGTCAAAA	*narK*	This study
narG-D	TAACGGATACGACCGACACA	*narH*	This study
narZ-U	TTACAACGCCTGCATCTCTG	*narU*	This study
narZ-D	TTTTCTGACTTGCCGCTTTT	*narY*	This study

*Δ*cydB*::*kan* and Δ*narZ*::*kan* Keio mutants were transformed with pCP20, which shows thermal induction of flippase (FLP) synthesis, to excise the kanamycin resistance cassette (KanR) via the flanking flippase recognition target (FRT) sites while maintaining the gene deletion (Datsenko & Wanner 2000). Individual colonies were streaked on kanamycin (25 μg/ml), ampicillin (200 μg/ml), and chloramphenicol (20 μg/ml) LB agar plates and selected, followed by PCR using the k2 and kt primers to confirm loss of the KanR cassette and pCP20 (Datsenko & Wanner 2000). Resulting kanamycin-susceptible mutants were then transduced with P1 phage stocks generated from the desired Keio mutant (Δ*cydD*::*kan* and Δ*narG*::*kan*) to incorporate the second mutation. Four PCR reactions were then performed to verify that the Δ*cydB*-Δ*cydD*::*kan* and Δ*narZ*-Δ*narG*::*kan* mutants had the correct structure. New junctions created between the KanR cassette and neighboring upstream and downstream sequences were verified using the kanamycin (kt or k2) and locus-specific (U or D) primers. A third reaction was carried out using the flanking locus-specific primers (U and D) to verify simultaneous loss of the parental (non-mutant) fragment and gain of the new mutant-specific fragment. A fourth reaction using primers k2 and kt was then used to screen for the KanR cassette.

Table C.3: Primers designed against the *Escherichia coli* BW25113 genome and used to contruct the Δ(*cyoA*-*cyoB*)::*kan,* Δ(*napA*-*napD*)::*kan,* and Δ*cydB*-Δ*cydD*-Δ(*cyoA*-*cyoB*)::*kan* mutants.*

Name	Sequence (5'-3')	Target gene	Reference
P1	ATTCCGGGGATCCGTCGACC	*kan*-FRT	Baba et al. 2006
P2	TGTAGGCTGGAGCTGCTTCG	*kan*-FRT	Baba et al. 2006
P1-rev	GGTCGACGGATCCCCGGAAT	*kan*-FRT	Baba et al. 2006
P2-rev	CGAAGCAGCTCCAGCCTACA	*kan*-FRT	Baba et al. 2006
cyoA-U	GCTGGATTATCTGGCGCTAC	*ampG*	This study
cyoB-D	GATGGAGCTGAACAACAGCA	*cyoC*	This study
napD-U	CGCAAAGCCAGTAACGGTAT	*napF*	This study
napA-D	AGCGGTAACACCTTGATTGC	*napG*	This study

*Δ(*cyoA*-*cyoB*)::*kan* and Δ(*napA*-*napD*)::*kan* mutants were generated by first performing three standard and one fusion PCR reaction to construct linear DNA fragments containing the KanR cassette and two flanking homology regions of the target genes. First, the KanR cassette was PCR amplified from Keio mutant genomic DNA using primers binding upstream (P1) and downstream (P2) of the flanking FRT sites. Junctions between the KanR cassette and neighboring upstream and downstream sequences for the target genes of interest were then PCR amplified using genomic DNA from the corresponding Keio mutants (Δ*cyoA*::*kan*, Δ*cyoB*::*kan*, Δ*napA*::*kan*, or Δ*napD*::*kan*) as template and primer pairs cyoA-U/P1-rev, cyoB-D/P2-rev, napA-D/P2-rev, or napD-U/P1-rev, respectively. Finally, PCR fragments from these reactions were assembled by fusion PCR using the flanking locus-specific primers sets cyoA-U/cyoB-D and napD-U/napA-D. Assembled PCR products were purified and electroporated into the parental BW25113 strain carrying plasmid pKD46 (Datsenko & Wanner 2000). Resulting mutants were selected on kanamycin (25 μg/ml) and ampicillin (100 μg/ml) LB agar plates to ensure loss of the pKD46 plasmid. New junctions created between the KanR cassette and neighboring upstream and downstream sequences were then verified for each mutation by PCR with kanamycin (kt or k2) and locus-specific (U or D) primers. The quadruple mutant Δ*cydB*-Δ*cydD*-Δ(*cyoA*-*cyoB*)::*kan* was generated by pCP20-mediated removal of the KanR cassette from the Δ*cydB*-Δ*cydD*::*kan* double mutant followed by transduction with the Δ(*cyoA*-*cyoB*)::*kan* mutation and PCR verification of the new mutant as described above.

C.2 Supplementary Figures

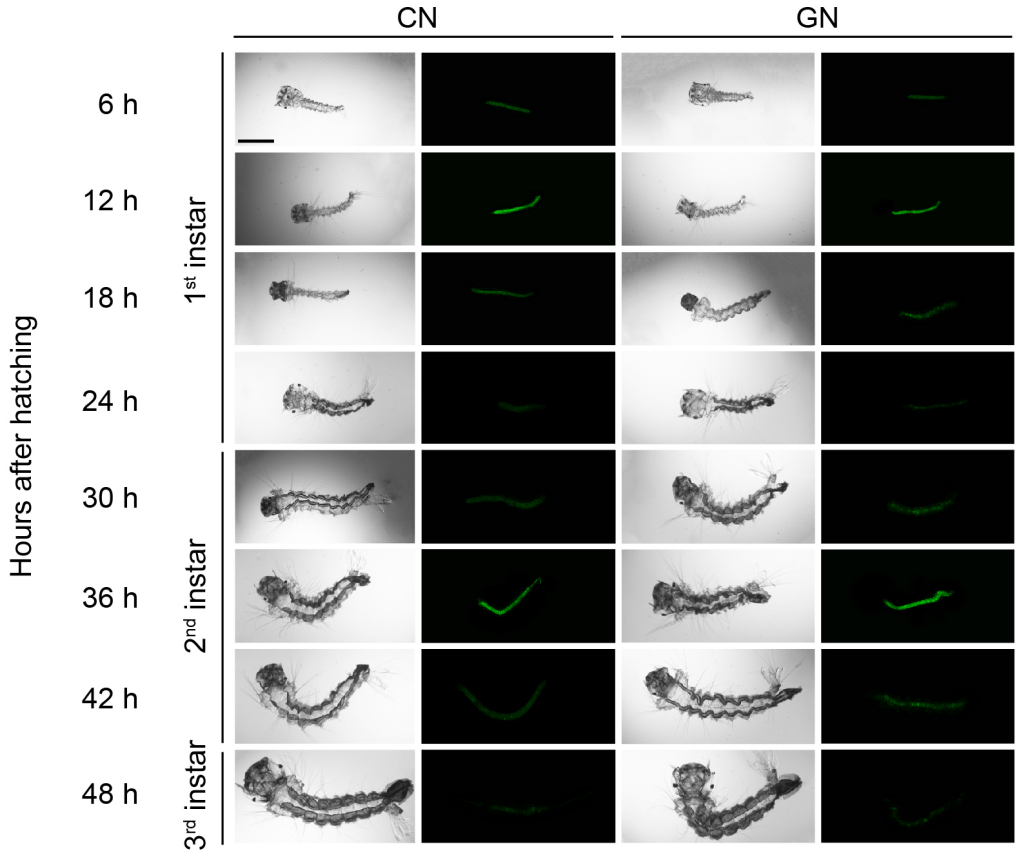


Figure C.1. Image iT fluorescence in conventional (CN) and gnotobiotic larvae inoculated with wild-type *Escherichia coli* (GN). Individual larvae were examined by confocal microscopy from 6-48 h post-hatching. Sampling times are indicated to the left. For each treatment, the image to the left shows a light image of a larva while the image to the right shows the corresponding fluorescence image. The anterior (head) of each larva is oriented to the left. Image iT fluorescence (green) is restricted to the midgut and is most intense in pre-critical size larvae (see main text). The scale bar in the upper left equals 100 μm. Ten individuals were examined for each treatment and time point with all outcomes similar to the images shown.

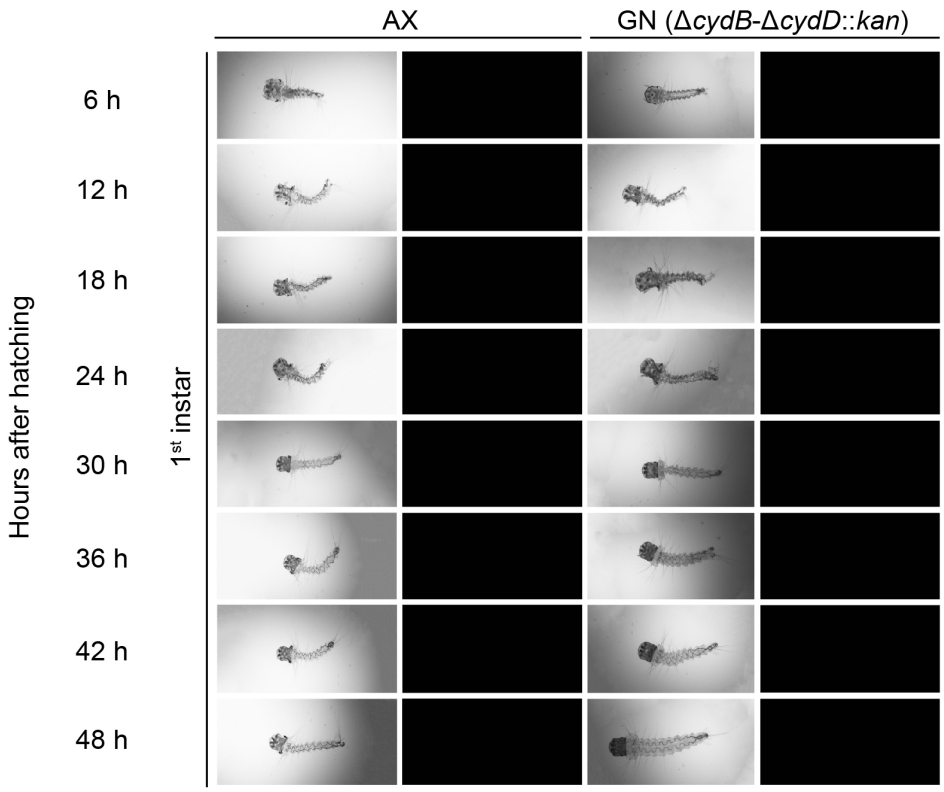


Figure C.2. Image iT fluorescence in axenic (AX) and gnotobiotic larvae inoculated with Δ*cydB*-Δ*cydD*::*kan* *Escherichia coli* (GN (Δ*cydB*-Δ*cydD*::*kan*)). Individual larvae were examined and are oriented as in Figure C.1. Note the absence of Image iT fluorescence in larvae for both treatments. Ten individuals were examined for each treatment and time point with all outcomes similar to the images shown.

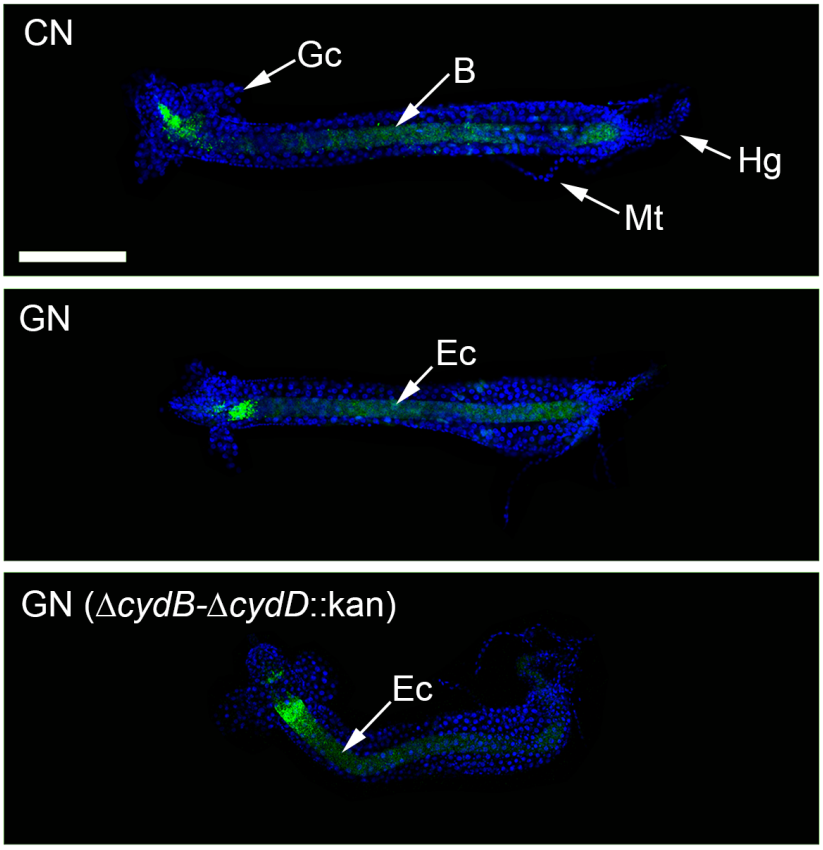


Figure C.3. Representative confocal microscopy images of digestive tracts from conventional (CN) and gnotobiotic larvae inoculated with wild-type (GN) or Δ*cydB*-Δ*cydD*::*kan Escherichia coli*. The foregut was removed in each image resulting in gastric caecae (Gc), malpighian tubules (Mt) and hindgut (Hg) being oriented left to right. Cell nuclei were stained with Hoechst 55532 (blue), while a peptidoglycan antibody (GeneTex) visualized by an Alexafluor 488 secondary antibody (green) labeled the mixed community of bacteria (B) in CN larvae and *E. coli* (Ec) in gnotobiotic larvae. Note the distribution and fluorescence intensity of bacteria is very similar across treatments. Methods for antibody labeling and confocal microscopy are as previously described (Vogel et al. 2017).

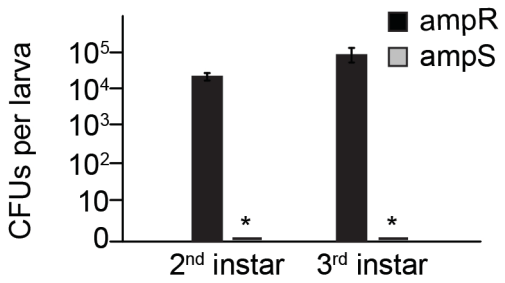


Figure C.4. Ampicillin treatment eliminated ampicillin-susceptible (ampS) *Escherichia coli* from *Aedes aegypti* larvae while ampicillin-resistant (ampR) *E. coli* persisted. Gnotobiotic larvae inoculated with ampR and ampS *E. coli* were treated by adding ampicillin to cultures after molting to the second (2^{nd}) or third (3^{rd}) instar followed homogenization 24 h post-treatment and culture on LB plates at 37 °C for 24 h to determine the number of colony-forming units (CFUs) ± SE per larva. Four individual larvae were bioassayed per treatment.

APPENDIX D

SUPPORTING INFORMATION FOR CHAPTER 5

D.1 Supplementary Tables

Table D.1: Bacterial isolates used in the study.

Mosquito species isolate was collected from	**Colony color and morphology**	**Culture medium**	**Genus**
Aedes aegypti	White/pink + sec	Nutrient agar (BHI)	*Paenibacillus*
Ae. aegypti	Yellow	Nutrient agar (BHI)	*Chryseobacterium*
Ae. aegypti	White	Nutrient agar (BHI)	*Sphingobacterium*
Ae. aegypti	White (small)	Blood agar (TSA base)	*Microbacterium*
Ae. aegypti	White (small)	Nutrient agar (BHI)	*Leucobacter*
Ae. atropalpus	Green	R2A	*Aquitalea*
Ae. atropalpus	White (small) + sec	R2A	*Comamonas*

Table D.2: Primers designed and used during the study.

Name	Sequence	Target gene	Target	Tm (°C)	Reference
General primers (product > 300 bp)					
27F	GAGAGTTTGATCCTGGCTCAG	16S rRNA	Most bacteria	50	Lane 1991
1492R	GGTTACCTTGTTACGACTT	16S rRNA	Most bacteria	50	Lane 1991
1492F	AAGTCGTAACAAGGTAACC	16S rRNA	Most bacteria	55	Lane 1991
129R	GGTTBCCCCATTCRG	23S rRNA	Most bacteria	55	Gurtler & Stanisich 1996
Quantitative PCR primers (product < 300 bp)					
*HDA1	ACTCCTACGGGAGGCAGCAGT	16S rRNA	Most bacteria	55	Walter et al. 2000
*HDA2	GTATTACCGCGGCTGCTGGCA	16S rRNA	Most bacteria	55	Walter et al. 2000
Aqui-qF	GTTGAGCACTCTAATGGGAC	16S-23S ITS	*Aquitalea* spp.	55	
Aqui-qR	TCGGTTTTATGAGATTGGCT	16S-23S ITS	*Aquitalea* spp.	55	
Chryseo-qF	GAAACTGCCATTGATACTGC	16S-23S ITS	*Chryseobacterium* spp.	55	
Chryseo-qR	CCCAAAAACGAGTTAGCATC	16S-23S ITS	*Chryseobacterium* spp.	55	
Coma-qF	GAGTCGAAAATCAGCGTTGC	16S-23S ITS	*Comamonas* spp.	55	
Coma-qR	GCACAAGAACCCAAGCAACT	16S-23S ITS	*Comamonas* spp.	55	
Micro-qF	AACTAGTTGTGGGGTCCA	16S-23S ITS	*Microbacterium* spp.	55	
Micro-qR	TGTATGTCAAGCCTTGGTAA	16S-23S ITS	*Microbacterium* spp.	55	
Paeni-qF	TAGGTGTTGGGGATTCGAT	16S-23S ITS	*Paenibacillus* spp.	55	
Paeni-qR	CACCTGTCTCCTCTGTCC	16S-23S ITS	*Paenibacillus* spp.	55	

*Universal primers were used for qPCR analysis of *Sphingobacterium*- and *Leucobacter*-associated mosquitoes.

D.2 Supplementary Figures

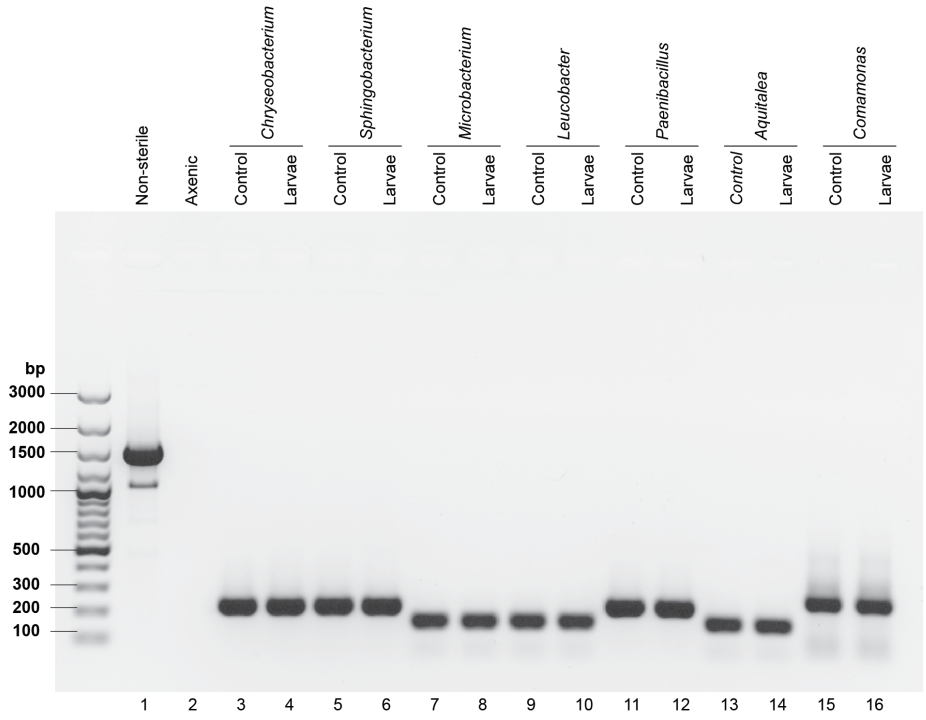


Figure D.1: Axenic *Aedes aegypti* and *Ae. atropalpus* first instars rapidly ingested bacteria. Conventional first instars were hatched in open containers containing distilled water and sterilized diet (Non-sterile). Axenic first instars were hatched in fully sterile conditions and fed sterilized diet (Axenic). Other axenic larvae were fed sterilized diet plus the indicated bacterial isolate. For each treatment, DNA was isolated from a pooled sample of 10 larvae that was collected 6 h post-inoculation after repeated washing and surface sterilization per Coon et al. (2014). DNA was also isolated from cultures of each bacterial isolate. DNA samples were then used as templates with universal or genus-specific primers (see 'Methods' and Table D.2). The agarose gel shows ethidium bromide stained PCR products. Lane 1, molecular mass marker labeled in base pairs (bp); Lane 2, universal primers plus DNA from conventional larvae; Lane 3, universal primers plus DNA from axenic larvae; Lanes 3-4, *Chryseobacterium*-specific primers plus template from *Chryseobacterium* (Control) or axenic first instars inoculated with *Chryseobacterium* (Larva). The same treatments are then shown for *Sphingobacterium* (Lanes 5-6), *Microbacterium* (Lanes 7-8), *Leucobacter* (Lanes 9-10), *Paenibacillus* (Lanes 11-12), *Aquitalea* (Lanes 13-14), and *Comamonas* (Lanes 15-16). This experiment was repeated four times with independently collected samples and each time yielded identical outcomes for both *Ae. aegypti* and *Ae. atropalpus*.

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