Published in final edited form as:

Mol Ecol. 2014 June ; 23(11): 2727–2739. doi:10.1111/mec.12771.

Mosquitoes rely on their gut microbiota for development

Kerri L. Coon, Kevin J. Vogel, Mark R. Brown, and Michael R. Strand

Department of Entomology, The University of Georgia, Athens, GA, 30602, USA

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, *Georgacraigius atropalpus*, is autogenous and produces eggs without blood feeding. Each mosquito species contained a low diversity community comprised primarily of aerobic bacteria acquired primarily from the aquatic habitat in which larvae developed. Our results suggested the communities in *Ae. aegypti* and *An. gambiae* larvae share more similarities with one another than with *Ge. 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 three mosquito species spanning the breadth of the Culicidae depend on their gut microbiome for development.

1. 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

Corresponding author: Michael R. Strand, Department of Entomology, University of Georgia, 120 Cedar Street, 420 Biological Sciences, Athens, GA 30602, USA, Fax: 706-542-2279, mrstrand@uga.edu.

K.L.C., K.J.V., M.R.B., and M.R.S. designed research, performed research, analyzed data and wrote the manuscript.

Data Accessibility:

Raw 454 reads were deposited in the NCBI Sequence Read Archive (<http://www.ncbi.nlm.nih.gov/sra>) under project ID PRJNA234011.

Sanger-sequenced 16S reads were assigned GenBank accessions KJ192338-KJ192343.

QIIME Input files, larval feeding data using fluorescently-labeled *E. coli* with R-code for statistical analysis, plus development and survival data for axenic, gnotobiotic, and conventional mosquitoes: Dryad doi:10.5061/dryad.s6223.

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; Gusmao *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 among 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 anti-parasite 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 bacterial gut 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* sp. (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 Anophelinae and Culicinae, which diverged 145–200 million years ago (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, *Georgacraigius 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 *Ge.*

atropalpus. Our results also showed that axenic larvae cannot develop but several community members and *Escherichia coli* rescued development.

2. Materials and methods

(a) Conventionally-reared mosquitoes

Ae. aegypti, *An. gambiae* and *Ge. 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 aluminum 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*. *Ge. atropalpus* females thereafter laid a first clutch of eggs on filter paper. Adult female *Ae. aegypti* and *An. gambiae* were blood fed two days post-emergence on an anesthetized rat until engorged. Females then laid a clutch of eggs approximately 36 h later on filter paper. Eggs from each species were stored in humidified containers at room temperature until needed.

(b) DNA extraction

Total genomic DNA was isolated from water in rearing pans containing conventionally reared mosquito larvae of each species that had molted to the final (fourth) instar (Figure 1). DNA was also isolated from 40 fourth instars from the same rearing pans (Figure 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*: 1) 80 non-blood fed (NBF) adults, which emerged from surface-sterilized pupae, 2) 40 three-day-old adults, which emerged from surface-sterilized pupae that blood fed (BF) on a surface sterilized host 24 h previously, 3) egg masses oviposited onto sterile paper in sterile water by females from surface-sterilized pupae (STR), and 4) egg masses oviposited by conventionally reared females (ConR) (Figure 1). Pupae produced from conventionally reared larvae were surface sterilized by placing in 2% bleach for 20 min and rinsing 3x in sterile H₂O. 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 sterile water source for two 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.

(c) 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 S1). Amplicons were generated from each sample in four separate 20 µl PCR reactions that were pooled prior to purification. Each reaction contained iProof High Fidelity polymerase (BioRad), 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); sequences 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 300–321 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 analyzed using the Quantitative Insights Into Microbial Ecology (QIIME) pipeline (Lozupone & Knight, 2005; DeSantis *et al.* 2006; Wang *et al.* 2007; Caporaso *et al.* 2010a; Caporaso *et al.* 2010b; 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 5 reads, they were omitted from downstream analyses. Chao1 and Shannon diversity indices were calculated for each sample as part of the QIIME analysis.

(d) Isolation of bacterial community members and taxonomic assignment

Ae. aegypti eggs, fourth instars, and adults (NBF and BF) from our conventional cultures were rinsed in 70% EtOH, transferred to 100 µl of sterile PBS 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 re-streaking onto fresh plates, followed by DNA isolation using the Gentra Puregene DNA isolation kit. Universal primers were used to amplify approximately 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 was 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, variable regions in intergenic spacers 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 S1) for a given isolate was verified by PCR using DNA from all other bacteria isolates cultured from mosquitoes.

(e) 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 3x in sterile H₂O. Ten eggs were placed in 25 cm² cell culture flasks (Corning) containing 20 ml of sterile H₂O. First instars hatched ca. 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 S1), which indicated no viable bacteria were present. Gnotobiotic larvae were generated by picking a colony of a given bacterial isolate (Table S2) and inoculating into 25 cm² 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 analyzed 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 axenic diet in water and incubated for 72 h at 28° C. The food mixture was then centrifuged at 3,000 rpm for 5 min followed by filter sterilization to produce bacteria-conditioned water. Axenic larvae were then placed in either: 1) conditioned water with or without new sterile diet or 2) conditioned water with sterile 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 analyzed 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/>).

(f) Larval feeding behavior

To monitor feeding by mosquito larvae, dye-based feeding assays were conducted using heat-killed *E. coli* labeled with fluorescein isothiocyanate (FITC) (Beck & Strand 2003) or sterile diet labeled for 1 h with acridine orange (AO) (200 µg/ml) in water. FITC-labeled bacteria (1×10^3 cfu/ul) were added to wells of a 24 well culture plate (Corning) containing 200 µl of water, 10–20 non-sterile or axenic larvae and standard diet (500 µg). AO-labeled

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.

3. Results

(a) 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 *Ae. aegypti*, *An. gambiae* and *Ge. 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 1). For *Ae. aegypti*, we also analyzed 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 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 1). These reads collapsed into 4,282, 9,229, and 6,998 non-redundant reads, which were unevenly distributed among samples (Table 1). After demultiplexing, sequences were grouped by percent similarity to form operational taxonomic units (OTUs) at a cutoff threshold of 97%. OTUs represented by 5 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 1). Rarefaction analyses tended toward saturation but did not fully plateau for most samples indicating that some community members were missed in our analyses (Figure S1). 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 S1). 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 1). The relatively low number of OTUs detected in mosquito samples also indicated that bacterial community diversity was low.

(b) Bacterial communities in *Ae. aegypti* and *An. gambiae* larvae differ from *Ge. 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). 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 *Ge. atropalpus* larvae (Figure 2). We detected the same bacterial families in water and larvae, but their relative abundance differed (Figure 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. *Ge. atropalpus* larvae in contrast contained a community dominated by Betaproteobacteria belonging to the Procabacteriaceae, 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 3A). In contrast, approximately half of the OTUs in *An. gambiae* and *Ge. atropalpus* larvae were unique (Figure 3B). *Ae. aegypti* larvae had a smaller proportion of unique OTUs (26%) (Figure 3B), but this was likely due to shallower sampling (Table 1). We also found that a majority of OTUs in larvae overlapped with the water where they developed (Figure 3C). We further examined community structure by performing a principal component analysis (PCA) on the Unifrac distances in each sample. Unifrac 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 among samples when only the presence or absence of bacterial taxa was considered (unweighted Unifrac distances) (Figure S2). This increased to 79% when taxon abundance was included (weighed Unifrac distances) (Figure S2). The water and larval communities of *Ae. aegypti* clustered with one another in our data sets but this clustering was lost for *Ge. atropalpus* and *An. gambiae* using weighted data (Figure S2). 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.

(c) Bacterial community composition changes during *Ae. aegypti* development

A few bacterial taxa are known to be transstadially transmitted in *Anopheles* sp. (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 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 immediately after emergence while BF adults were held in sterile chambers with only sterile water source available for consumption before blood feeding on a host. By taking 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 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). Blood feeding further reduced the abundance of dominant larval community members, while greatly increasing Comamonadaceae (Figure 2). The low number of reads from NBF adults resulted in their removal from PCA, though 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 blood meal (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 (Kittaypong *et al.* 2000), no sequences corresponding to *Wolbachia* were present in our pyrosequencing data sets, while PCR screening using specific primers (Table 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 *Ge. atropalpus* cultures, but bacteria do reside on the egg surface (Chao 1963). Pyrosequencing showed that egg clutches from conventionally reared *Ae. aegypti* harbored an abundance of Gammaproteobacteria (Pseudomonadaceae, Enterobacteriaceae and Aeromonadaceae) and to a lesser degree Flavobacteriaceae (Figure 2A). Eggs laid by females from sterilized pupae also had a high relative abundance of Gammaproteobacteria, primarily Pseudomonadaceae, plus Flavobacteriaceae and Betaproteobacteria (Figure 2A). However, only 7 OTUs in 4 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 S2). 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.

(d) 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 *Ge. atropalpus* first instars were placed in sterile water and fed sterilized diet, all died after 5 days without molting (Figure 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 μ l of water from our conventional cultures developed normally as did conventionally reared larvae fed sterilized diet (Figure 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.

(e) 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. Since 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 S2). 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 S1). 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 5). PCR assays of individual mosquitoes indicated that each bacterial strain except *Microbacterium* was present in larvae after molting to the fourth instar and also was present in newly emerged adults (Figure 5). Each strain detected in adults was also viable as evidenced by the ability to recover and culture from homogenates of adults.

Since multiple community members rescued development, we assessed whether a non-community species had the same effect. For this experiment we used the DH5 α strain of *E. coli* since 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 5). PCR data also confirmed that *E. coli* colonized axenic larvae and was transstadially transmitted to adults (Figure 6). Thus, several strains of bacteria colonized axenic *Ae. aegypti* and rescued development.

(f) 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 5A). Inoculating cultures of axenic larvae with each strain of bacterium in the absence of sterile standard diet also resulted in no

rescue, although ~80% molted 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 sterile diet died as first instars (Figure 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* labeled with FITC or sterilized diet labeled with AO. We then quantified consumption of each by measuring fluorescence in the gut of 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, molted, and developed into adults (Figure S3).

4. 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 (Bossiere *et al.* 2012; Osei-Poku *et al.* 2012). Wang *et al.* (2011) reported higher diversity communities in *An. 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 (*Ae. aegypti*, *Ge. 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; Bossiere *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.

Since 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 the aquatic habitat strongly influences the bacteria 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 *Ge. atropalpus*. With only three mosquito species, we recognize the functional significance of this result must be viewed cautiously. Nonetheless, they suggest 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 lab 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 molting and metamorphosis (Moll *et al.* 2001), our results indicate 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 blood meal are also consistent with patterns seen in other studies (Oliviera *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 *Aedes 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 *E. 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 bacteria species rescue development of gnotobiotic larvae. One option is one or more conserved bacterial products provide signaling 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 signaling 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 signaling (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 signaling 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).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

The authors thank J. Johnson for assistance with photography and helping establish protocols for producing axenic mosquitoes. We also thank A. Robertson for technical support and S.R. Robertson for assistance with mosquito colonies. This work was supported by the National Science Foundation (Graduate Research Fellowship 038550-04) (KLC), and the National Institutes of Health (R01 AI033108) (MRB, MRS) and (F32 GM109750) (KJV).

References

- Bahia AC, Dong Y, Blumberg BJ, et al. Exploring *Anopheles* gut bacteria for *Plasmodium* blocking activity. *Environmental Microbiology*. 2014;10.1111/1462-2920.12381
- Beck M, Strand MR. RNA interference silences *Microplitis demolitor* bracovirus genes and implicates *gcl1.8* in disruption of adhesion in infected host cells. *Virology*. 2003; 314:521–535. [PubMed: 14554081]
- Boissiere A, Tchioffo MT, Bachar D, et al. Midgut microbiota of the malaria mosquito vector *Anopheles gambiae* and interactions with *Plasmodium falciparum* infection. *PLoS Pathogens*. 2012; 8:e1002742. [PubMed: 22693451]
- Briegleb H. Physiological bases of mosquito ecology. *Journal of Vector Ecology*. 2003; 28:1–11. [PubMed: 12831123]
- Briones AM, Shililu J, Githure J, Novak R, Raskin L. *Thorsellia anophelis* is the dominant bacterium in a Kenyan population of adult *Anopheles gambiae* mosquitoes. *ISME Journal*. 2008; 2:74–82. [PubMed: 18180748]

- Caporaso JG, Bittinger K, Bushman FD, DeSantis TZ, Andersen GL, Knight R. PyNAST: a flexible tool for aligning sequences to a template alignment. *Bioinformatics*. 2010a; 26:266–267. [PubMed: 19914921]
- Caporaso JG, Kuczynski J, Stombaugh J, et al. QIIME allows analysis of high-throughput community sequencing data. *Nature Methods*. 2010b; 7:335–336. [PubMed: 20383131]
- Chao J, Wistreich GA, Moore J. Failure to isolate microorganisms from within mosquito eggs. *Annals of the Entomological Society of America*. 1963; 56:559–561.
- Chavshin AR, Oshaghi MA, Vatandoost H, et al. Identification of bacterial microflora in the midgut of the larvae and adult of wild caught *Anopheles stephensi*: a step toward finding suitable paratransgenesis candidates. *Acta Tropica*. 2012; 121:129–134. [PubMed: 22074685]
- Chouaia B, Rossi P, Montagna M, et al. Molecular evidence for multiple infections as revealed by typing of *Asaia* bacterial symbionts of four mosquito species. *Applied Environmental Microbiology*. 2010; 76:7444–7450. [PubMed: 20851960]
- Chouaia B, Rossi P, Epis S, et al. Delayed larval development in *Anopheles* mosquitoes deprived of *Asaia* bacterial symbionts. *BMC Microbiology*. 2012; 12:S2. [PubMed: 22375964]
- Cirimotich CM, Dong YM, Clayton AM, et al. Natural microbe-mediated refractoriness to *Plasmodium* infection in *Anopheles gambiae*. *Science*. 2011; 332:855–858. [PubMed: 21566196]
- Clements, AN. *The Biology of Mosquitoes*, Vol. 1. Development, Nutrition, and Reproduction. Chapman & Hall; New York: 1992.
- Cole JR, Wang Q, Cardenas E, et al. The Ribosomal Database Project: improved alignments and new tools for rRNA analysis. *Nucleic Acids Research*. 2009; 37:D141–D145. [PubMed: 19004872]
- Corby-Harris V, Pontaroli AC, Shinkets LJ, Bennetzen JL, Habel KE, Promislow DEL. Geographical distribution and diversity of bacteria associated with natural populations of *Drosophila melanogaster*. *Applied Environmental Microbiology*. 2007; 73:3470–3479. [PubMed: 17400769]
- Damiani C, Ricci I, Crotti E, et al. Mosquito-bacteria symbiosis: the case of *Anopheles gambiae* and *Asaia*. *Microbial Ecology*. 2010; 60:644–654. [PubMed: 20571792]
- DeMaio J, Pumpuni CB, Kent M, Beier JC. The midgut bacterial flora of wild *Aedes triseriatus*, *Culex pipiens*, and *Psorophora columbiae* mosquitoes. *The American Journal of Tropical Medicine and Hygiene*. 1996; 54:219–223. [PubMed: 8619452]
- DeSantis TZ, Hugenholtz P, Larsen N, et al. Greengenes, a chimera-checked 16S rRNA gene database and workbench compatible with ARB. *Applied Environmental Microbiology*. 2006; 72:5069–5072. [PubMed: 16820507]
- Djadjid ND, Jazayeri H, Raz A, Favia G, Ricci I, Zakeri S. Identification of the midgut microbiota of *An. stephensi* and *An. maculipennis* for their application as a paratransgenic tool against malaria. *PLoS One*. 2011; 6:e28484. [PubMed: 22163022]
- Dong YM, Manfredini F, Dimopoulos G. Implication of the mosquito midgut microbiota in defense against malaria parasites. *PLoS Pathogens*. 2009; 5:1000423.
- Edgar RC. Search and clustering orders of magnitude faster than BLAST. *Bioinformatics*. 2010; 26:2460–2461. [PubMed: 20709691]
- Engel P, Moran NA. The gut microbiota of insects-diversity in structure and function. *FEMS Microbiology Reviews*. 2013; 37:699–735. [PubMed: 23692388]
- Engel P, Martinson VG, Moran NA. Functional diversity within the simple gut microbiota of the honey bee. *Proceedings of the National Academy of Sciences USA*. 2012; 109:11002–11007.
- Favia G, Ricci I, Damiani C, et al. Bacteria of the genus *Asaia* stably associate with *Anopheles stephensi*, an Asian malarial mosquito vector. *Proceedings of the National Academy of Sciences USA*. 2007; 104:9047–9051.
- Foster WA. Mosquito sugar feeding and reproductive energetics. *Annual Review of Entomology*. 1995; 40:443–474.
- Gaio AD, Gusmão DS, Santos AV, Berbert-Molina MA, Pimenta PFP, Lemos FJA. Contribution of midgut bacteria to blood digestion and egg production in *Aedes aegypti* (Diptera: Culicidae) (L.). *Parasites & Vectors*. 2011; 4:105. [PubMed: 21672186]
- Gulia-Nuss M, Eum JH, Strand MR, Brown MR. Ovary ecdysteroidogenic hormone activates egg maturation in the mosquito *Georgacraigius atropalpus* after adult eclosion or a blood meal. *Journal of Experimental Biology*. 2012; 215:3758–3767. [PubMed: 22811249]

- Gusmão DS, Santos AV, Marini DC, Bacci M, Berbert-Molina MA, Lemos FJA. Culture-dependent and culture-independent characterization of microorganisms associated with *Aedes aegypti* (Diptera: Culicidae) (L.) and dynamics of bacterial colonization in the midgut. *Acta Tropica*. 2010; 115:275–281. [PubMed: 20434424]
- Haas BJ, Gevers D, Earl AM, et al. Chimeric 16S rRNA sequence formation and detection in Sanger and 454-pyrosequenced PCR amplicons. *Genome Research*. 2011; 21:494–504. [PubMed: 21212162]
- Katoh K, Kuma K, Toh H, Miyata T. MAFFT version 5: improvement in accuracy of multiple sequence alignment. *Nucleic Acids Research*. 2005; 33:511–518. [PubMed: 15661851]
- Kittayapong P, Baisley KJ, Baimai V, O'Neill SL. Distribution and diversity of *Wolbachia* infections in southeast asian mosquitoes (Diptera: Culicidae). *Journal of Medical Entomology*. 2000; 37:340–345. [PubMed: 15535575]
- Kunin V, Engelbrektson A, Ochman H, Hugenholtz P. Wrinkles in the rare biosphere: pyrosequencing errors can lead to artificial inflation of diversity estimates. *Environmental Microbiology*. 2010; 12:118–123. [PubMed: 19725865]
- Lee WJ, Brey PT. How microbiomes influence metazoan development: insights from history and *Drosophila* modeling of gut-microbe interactions. *Annual Review of Cell Biology*. 2013; 29:571–592.
- Lindh JM, Borg-Karlson A-K, Faye I. Transstadial and horizontal transfer of bacteria within a colony of *Anopheles gambiae* (Diptera: Culicidae) and oviposition response to bacteria-containing water. *Acta Tropica*. 2008; 107:2421–250.
- Lozupone C, Knight R. UniFrac: a new phylogenetic method for comparing microbial communities. *Applied Environmental Microbiology*. 2005; 71:8228–8235. [PubMed: 16332807]
- Lozupone CA, Hamady M, Kelley ST, Knight R. Quantitative and qualitative beta diversity measures lead to different insights into factors that structure microbial communities. *Applied Environmental Microbiology*. 2007; 73:1576–1585. [PubMed: 17220268]
- Martinson VG, Danforth BN, Minckley RL, Rueppell O, Tingek S, Moran NA. A simple and distinctive microbiota associated with honey bees and bumble bees. *Molecular Ecology*. 2011; 20:619–628. [PubMed: 21175905]
- McDonald D, Price MN, Goodrich J, et al. An improved Greengenes taxonomy with explicit ranks for ecological and evolutionary analyses of bacteria and archaea. *ISME Journal*. 2012; 6:610–618. [PubMed: 22134646]
- McMeniman CJ, Lane RV, Cass BN, et al. Stable introduction of a life-shortening *Wolbachia* infection into the mosquito *Aedes aegypti*. *Science*. 2009; 323:141–144. [PubMed: 19119237]
- Merritt RW, Dadd RH, Walker ED. Feeding behavior, natural food, and nutritional relationships of larval mosquitos. *Annual Review of Entomology*. 1992; 37:349–376.
- Moll RM, Romoser WS, Modrzakowski MC, Moncayo AC, Lerdthusnee K. Meconial peritrophic membranes and the fate of midgut bacteria during mosquito (Diptera: Culicidae) metamorphosis. *Journal of Medical Entomology*. 2001; 38:29–32. [PubMed: 11268687]
- Moran NA. Symbiosis. *Current Biology*. 2006; 16:R866–R871. [PubMed: 17055966]
- Oliveira JHM, Goncalves RLS, Lara FA, et al. Blood meal-derived heme decreases ROS levels in the midgut of *Aedes aegypti* and allows proliferation of intestinal microbiota. *PLoS Pathogens*. 2011; 7:1001320.
- Osei-Poku J, Mbogo CM, Palmer WJ, Jiggins FM. Deep sequencing reveals extensive variation in the gut microbiota of wild mosquitoes from Kenya. *Molecular Ecology*. 2012; 21:5138–5150. [PubMed: 22988916]
- Price MN, Dehal PS, Arkin AP. FastTree 2-approximately maximum-likelihood trees for large alignments. *PLoS One*. 2010; 5:e9490. [PubMed: 20224823]
- Pumpuni CB, Demaio J, Kent M, Davis JR, Beier JC. Bacterial population dynamics in three anopheline species: the impact on *Plasmodium* sporogonic development. *American Journal of Tropical Medicine and Hygiene*. 1996; 54:214–218. [PubMed: 8619451]
- Reidenbach KR, Cook S, Bertone MA, Harbach RE, Wiegmann BM, Besansky NJ. Phylogenetic analysis and temporal diversification of mosquitoes (Diptera: Culicidae) based on nuclear genes and morphology. *BMC Evolutionary Biology*. 2009; 9:298. [PubMed: 20028549]

- Riehle MA, Brown MR. Insulin receptor expression during development and a reproductive cycle in the ovary of the mosquito *Aedes aegypti*. *Cell & Tissue Research*. 2002; 308:409–420. [PubMed: 12107434]
- Sanders JG, Powell S, Kronauer DJC, Vasconcelos HL, Frederickson ME, Pierce NE. Stability and phylogenetic correlation in gut microbiota: lessons from ants and apes. *Molecular Ecology*. 2014
- Shin SC, Kim SH, You H, et al. *Drosophila* microbiome modulates host developmental and metabolic homeostasis via insulin signaling. *Science*. 2011; 334:670–674. [PubMed: 22053049]
- Staubach F, Baines JF, Kunzel S, Bik EM, Petrov DA. Host species and environmental effects on bacterial communities associated with *Drosophila* in the laboratory and in the natural environment. *PLoS One*. 2013; 8:e70749. [PubMed: 23967097]
- Storelli G, Defaye A, Erkosar B, Hols P, Royet J, Leulier F. *Lactobacillus plantarum* promotes *Drosophila* systemic growth by modulating hormonal signals through TOR-dependent nutrient sensing. *Cell Metabolism*. 2011; 14:403–414. [PubMed: 21907145]
- Terenius O, Lindh JM, Eriksson-Gonzales K, et al. Midgut bacterial dynamics in *Aedes aegypti*. *FEMs Microbial Ecology*. 2012; 80:556–565.
- Wang Q, Garrity GM, Tiedje JM, Cole JR. Naive Bayesian classifier for rapid assignment of rRNA sequences into the new bacterial taxonomy. *Applied Environmental Microbiology*. 2007; 73:5261–5267. [PubMed: 17586664]
- Wang Y, Gilbreath TM, Kukutla P, Yan GY, Xu JN. Dynamic gut microbiome across life history of the malaria mosquito *Anopheles gambiae* in Kenya. *PLoS One*. 2011; 6:e24767. [PubMed: 21957459]
- Wong ACN, Ng P, Douglas AE. Low diversity bacterial community in the gut of the fruitfly *Drosophila melanogaster*. *Environmental Microbiology*. 2011; 13:1889–1900. [PubMed: 21631690]
- Wotton RS, Chaloner DT, Yardley CA, Merritt RW. Growth of *Anopheles* mosquito larvae on dietary microbiota in aquatic surface microlayers. *Medical & Veterinary Entomology*. 1997; 11:65–70. [PubMed: 9061679]
- Xi Z, Ramirez JL, Dimopoulos G. The *Aedes aegypti* toll pathway controls dengue virus infection. *PLoS Pathogens*. 2008; 4:e1000098. [PubMed: 18604274]
- Ye YXH, Woolfit M, Huttley GA, et al. Infection with a virulent strain of *Wolbachia* disrupts genome wide-patterns of cytosine methylation in the mosquito *Aedes aegypti*. *PLoS One*. 2013; 8:e66482. [PubMed: 23840485]
- Zhang GM, Hussain M, O'Neill SL, Asgari S. *Wolbachia* uses a host microRNA to regulate transcripts of a methyltransferase, contributing to dengue virus inhibition in *Aedes aegypti*. *Proceedings of the National Academy of Sciences, USA*. 2013; 110:10276–10281.

Figure 1.

Life cycle of *Ae. aegypti*, *An. gambiae* and *Ge. 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. *Ge. atropalpus* are autogenous and oviposit a first clutch of eggs without taking a blood meal. 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 *Ge. 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 NBF adults and 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).

Figure 2.

Bacterial composition at the phylum and family levels in laboratory reared *Ae. aegypti*, *An. gambiae* and *Ge. atropalpus*. Water and larval samples (fourth instars) were analyzed for each species. Adult and egg samples were analyzed 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.

Figure 3.

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

Figure 4.

Survival of first instar *Ae. aegypti*, *An. gambiae*, and *Ge. 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 performed by Tukey-Kramer HSD (NS, treatments not significantly different; ***, Axenic treatment significantly different from the With Bacteria and Non-sterile treatments, $p < 0.0001$).

Figure 5.

Several bacterial isolates from *Ae. aegypti* and *E. 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 per treatment. 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 among treatments.

Figure 6.

Most bacterial isolates colonized axenic *Ae. 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), *E. 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.

Table 1

Sequencing statistics from 454 pyrotagging experiments

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

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