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Mosquitoes rely on their gut microbiota for development

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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

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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

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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 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 Anophilinae and Culicinae, which diverged 145–200 Ma (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.

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 (Fig. 1). DNA was also isolated from 40 fourth instars from the same rearing pans (Fig. 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 that 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) (Fig. 1). Pupae produced from conventionally reared larvae were surface-sterilized by placing in 2% bleach for 2 min and rinsing 3× in sterile H_2O. 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

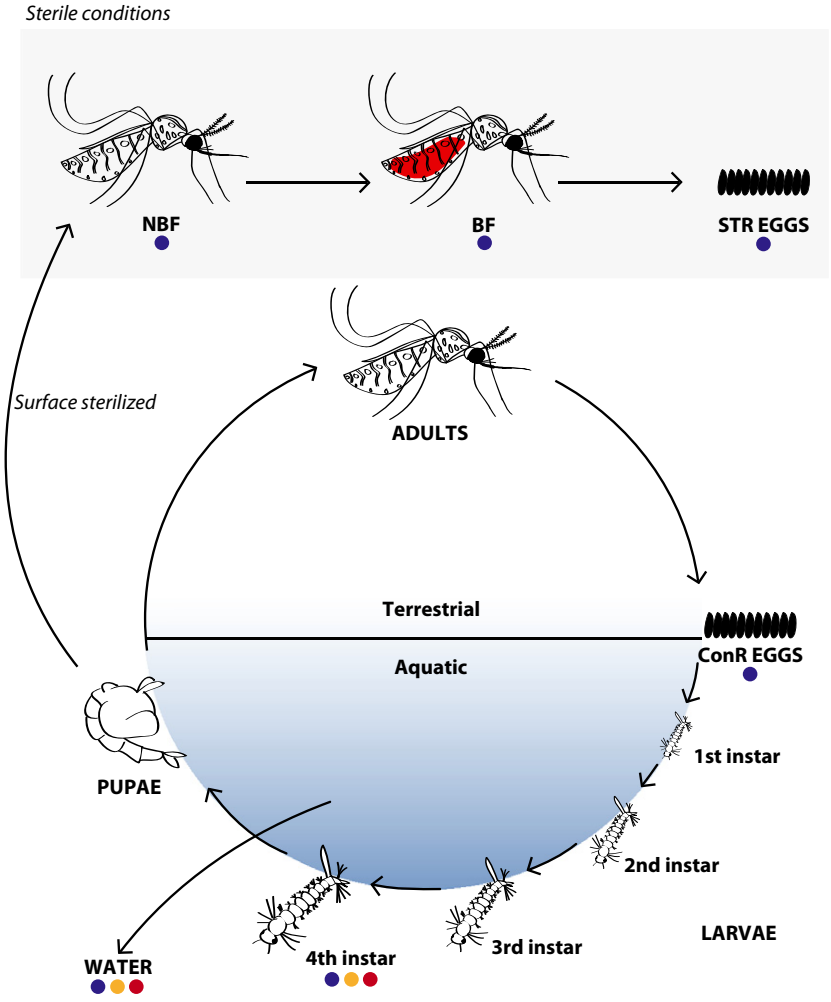

Fig. 1 Life cycle of *Aedes aegypti*, *Anopheles gambiae* and *Georgecraigius atropalpus* and samples collected for 454 pyrotagging analysis. Each species oviposits eggs that hatch in aquatic habitats where the larval-stage feeds and acquires bacteria that colonize the digestive tract. Larvae undergo 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 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).

also collected immediately after emergence, held in sterile cages and provided a 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 eggs 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 S1, Supporting information). 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); 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 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 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 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 S1, Supporting information) for a given isolate was verified by PCR using DNA from all other bacteria 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 3× in sterile H_2O. Ten eggs were placed in 25-cm^2 cell culture flasks (Corning) containing 20 ml of sterile H_2O. 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 S1, Supporting information), which indicated no viable bacteria were present. Gnotobiotic larvae were generated by picking a colony of a given bacterial isolate (Table S2, Supporting information) 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 axenic diet in water and incubated for 72 h at

28 °C. The food mixture was then centrifuged at 835 *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 sterile diet or (ii) 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 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 sterile diet labelled for 1 h with acridine orange (AO) (200 μg/ml) in water. FITC-labelled bacteria (1×10^3 cfu/ul) were added to wells of a 24-well culture plate (Corning) containing 1 ml of water, 10–20 nonsterile or axenic larvae and standard diet (500 μg). AO-labelled diet was added to cultures containing nonsterile 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.

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 (Fig. 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) (Fig. 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 4282, 9229 and 6998 nonredundant reads, which were unevenly distributed among samples (Table 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 1). Rarefaction analyses tended towards saturation, but did not fully plateau for most samples, indicating that some community members were missed in our analyses (Fig. S1, Supporting information). 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 (Fig. S1, Supporting information). Taken together, mosquito samples contained fewer OTUs than detected in water,

Table 1 Sequencing statistics from 454 pyrotagging experiments

Sample	Filtered reads (% of total reads)	Nonredundant reads	OTUs >5 reads
Run 1	33 806 (62.8)	4282	—
Aedes aegypti water	12 231	2535	123
Ae. aegypti larvae	5131	615	66
Ae. aegypti NBF adults	713	168	35
Ae. aegypti BF adults	15 731	964	22
Run 2	77 430 (60.4)	9229	—
Georgecraigius atropalpus water	12 986	1906	144
Anopheles gambiae water	22 781	3178	161
Ae. aegypti eggs (ConR)*	28 150	2960	72
Ae. aegypti eggs (STR)*	13 513	1185	53
Run 3	85 398 (69.2)	6998	—
G. atropalpus larvae	26 064	2331	91
An. gambiae larvae	59 334	4667	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.

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.

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 (Fig. 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 (Fig. 2). We detected the same bacterial families in water and larvae, but their relative abundance differed (Fig. 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 (Fig. 3A). In contrast, approximately half of the OTUs in *An. gambiae* and *G. atropalpus* larvae were unique (Fig. 3B). *Ae. aegypti* larvae had a smaller proportion of unique OTUs (26%) (Fig. 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 (Fig. 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

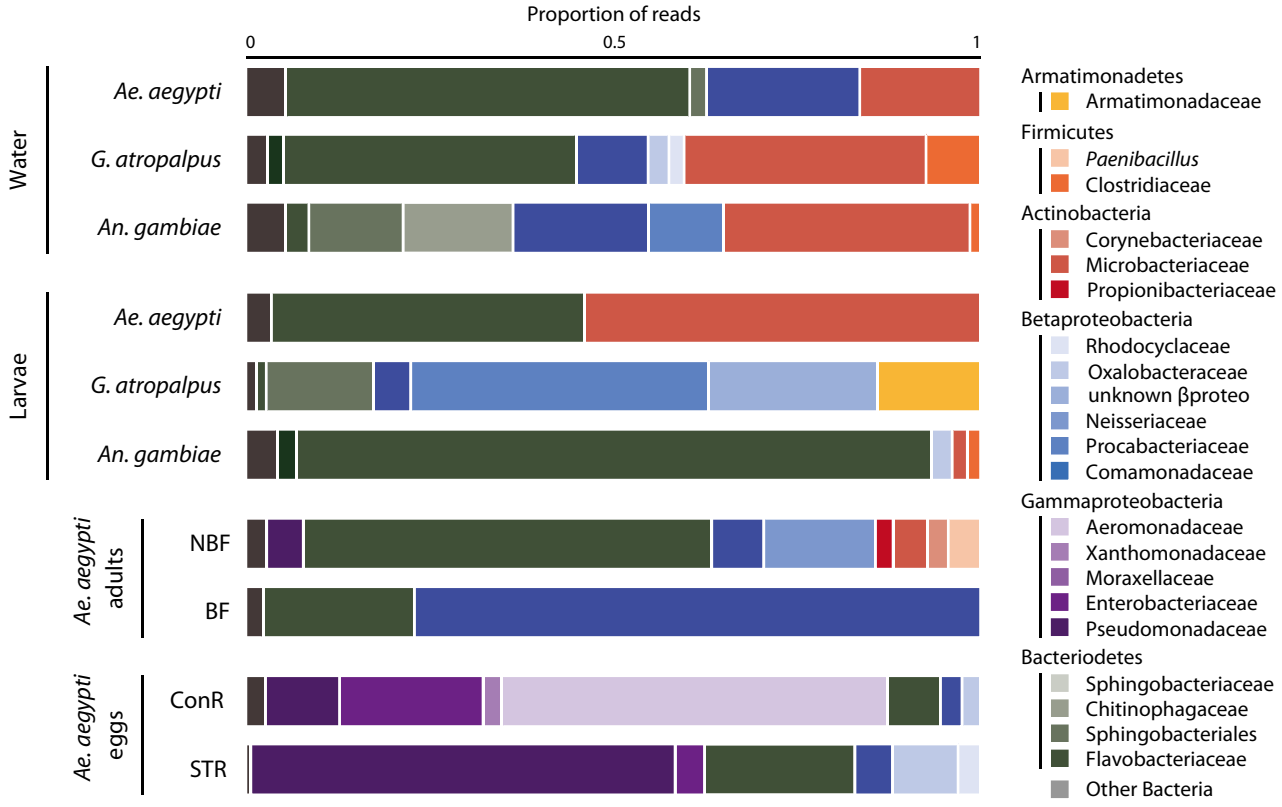

Fig. 2 Bacterial composition at the phylum and family levels in laboratory-reared *Aedes aegypti*, *Anopheles gambiae* and *Georgecraigius atropalpus*. Water and larval samples (fourth instars) were analysed for each species. Adult and egg samples were analysed for only *Ae. aegypti*. Each bar graph 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.

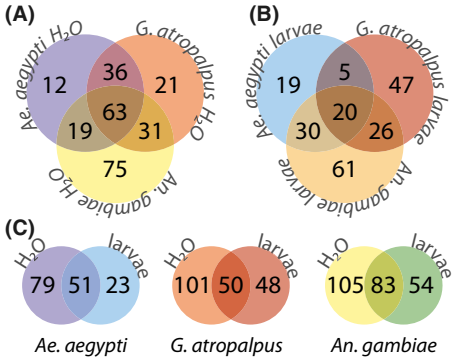

Fig. 3 Overlap between OTUs in (A) the water habitat larvae of each mosquito species was reared in, (B) the larval stage of each species and (C) water and larvae for each species.

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) (Fig. S2, Supporting information). This increased to 79% when taxon abundance was included (weighed Unifrac distances) (Fig. S2, Supporting information). 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 (Fig. S2, Supporting information). 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* 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 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 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 (Fig. 2). Blood feeding further reduced the abundance of dominant larval community members, while greatly increasing Comamonadaceae (Fig. 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 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 (Fig. 2A). Eggs laid by females from sterilized pupae also had a high relative abundance of Gammaproteobacteria, primarily Pseudomonadaceae, plus Flavobacteriaceae and Betaproteobacteria (Fig. 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 (Fig. S2, Supporting information). 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 nonsterilized 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 nonsterilized 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 (Fig. 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 (Fig. 4). Second, axenic larvae fed sterilized fish food, which is also commonly used to rear mosquitoes, failed to develop, while nonsterile larvae fed sterilized fish food developed normally.

Several bacterial community members rescue development

We used *Ae. aegypti* to determine whether any members of its microbiome could colonize axenic larvae and

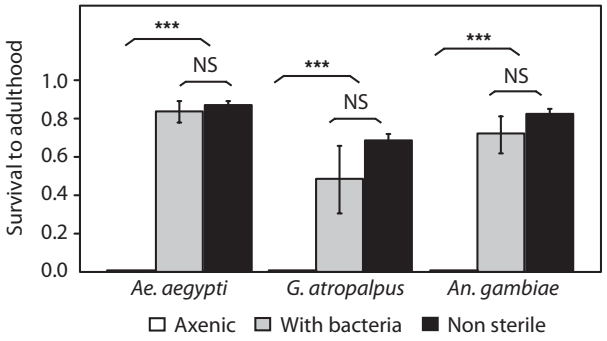

Fig. 4 Survival of first-instar *Aedes aegypti*, *Anopheles gambiae* and *Georgecraigius atropalpus* to adulthood under three conditions: axenic larvae fed sterilized diet (Axenic); axenic larvae fed sterilized diet plus bacteria from the conventional laboratory habitat (With bacteria), and conventional, nonsterile larvae fed sterilized diet (nonsterile). 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 nonsterile treatments, $P < 0.0001$).

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 S2, Supporting information). 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, Supporting information). 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 (Fig. 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 (Fig. 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 noncommunity 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

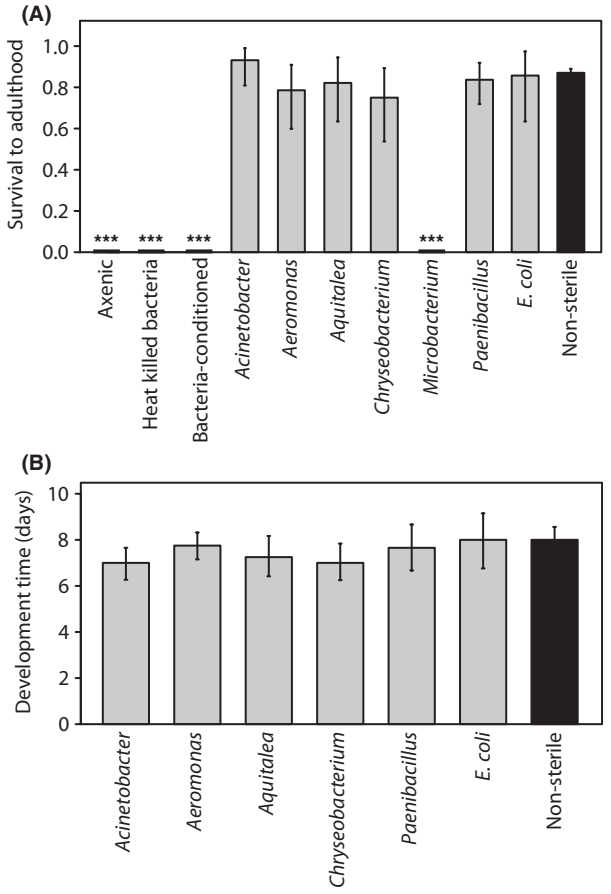

Fig. 5 Several bacterial isolates from *Aedes aegypti* and *Escherichia coli* rescue development of axenic larvae. (A) Proportion of axenic first instars that developed into adults when fed: sterilized diet only (Axenic), heat-killed bacteria plus sterilized diet (Heat-killed bacteria), sterilized diet in bacteria-conditioned water (Bacteria conditioned) or sterilized diet plus different bacterial isolates. Nonsterile 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. Nonsterile larvae served as the positive control. Columns present mean values with 95% confidence intervals for each treatment. ANOVA detected no differences between treatments.

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 (Fig. 5). PCR data also confirmed that *E. coli* colonized axenic larvae and was transstadially transmitted to adults (Fig. 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 (Fig. 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% 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 sterile diet died as first instars (Fig. 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 nonsterile *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 individual larvae at 2-h intervals for 8 h. Our results showed that axenic and nonsterile first instars consumed dead bacteria and axenic diet at similar rates but only the latter grew, moulted and developed into adults (Fig. S3, Supporting information).

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

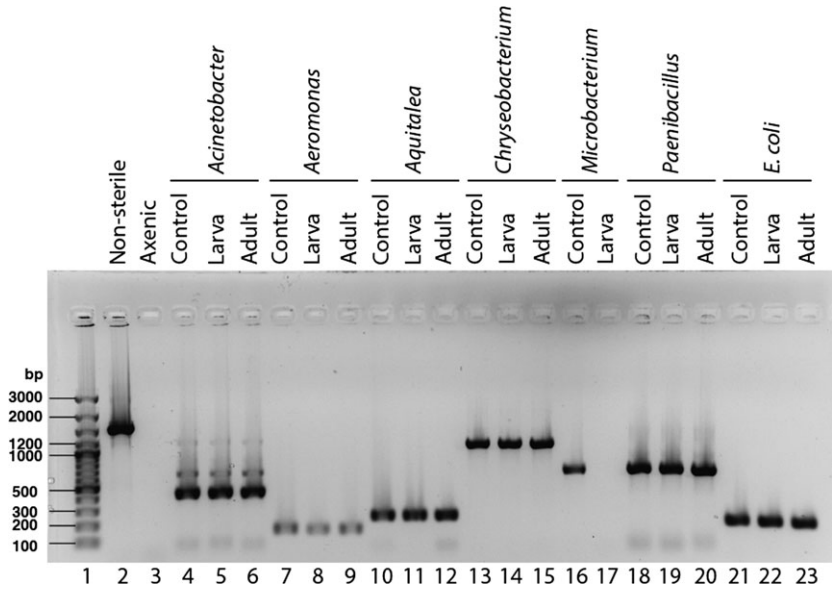

Fig. 6 Most bacterial isolates colonized axenic *Aedes aegypti*. Axenic first instars were fed sterilized diet plus the indicated bacterial isolate. DNA was then isolated from an individual and used as template with universal or taxon-specific primers. The agarose gel shows ethidium bromide-stained PCR 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.

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 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 *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 nonsterile 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 bacteria 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).

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K.L.C, K.J.V., M.R.B. and M.R.S. designed research, performed research, analysed 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 accession no. 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.

Supporting information

Additional supporting information may be found in the online version of this article.

Fig. S1 Rarefaction data from pyrotagging experiments.

Fig. S2 Principal components analysis of (A) weighted and (B) unweighted UniFrac distances of the samples.

Fig. S3 Axenic and non-sterile *Ae. aegypti* larvae consume dead bacteria and diet.

Table S1. Primers designed and used during the study.

Table S2. Bacterial taxa isolated, cultured and identified from *Ae. aegypti*.