

## MICROBIAL ECOLOGY

# Carryover effects of larval exposure to different environmental bacteria drive adult trait variation in a mosquito vector

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Conditions experienced during larval development of holometabolous insects can affect adult traits, but whether differences in the bacterial communities of larval development sites contribute to variation in the ability of insect vectors to transmit human pathogens is unknown. We addressed this question in the mosquito *Aedes aegypti*, a major arbovirus vector breeding in both sylvatic and domestic habitats in Sub-Saharan Africa. Targeted metagenomics revealed differing bacterial communities in the water of natural breeding sites in Gabon. Experimental exposure to different native bacterial isolates during larval development resulted in significant differences in pupation rate and adult body size but not life span. Larval exposure to an Enterobacteriaceae isolate resulted in decreased antibacterial activity in adult hemolymph and reduced dengue virus dissemination titer. Together, these data provide the proof of concept that larval exposure to different bacteria can drive variation in adult traits underlying vectorial capacity. Our study establishes a functional link between larval ecology, environmental microbes, and adult phenotypic variation in a holometabolous insect vector.

## INTRODUCTION

For many holometabolous insects (that is, with complete metamorphosis), the ecological niche of larval stages differs greatly from that of adults. For example, mosquito larvae develop in aquatic habitats, whereas the adults live in terrestrial habitats. Holometabolism allows larvae and adults of the same species to exploit different resources and avoid intraspecific competition (1). However, larval and adult stages of holometabolous insects are not independent from each other, because the biotic and abiotic larval environment can influence adult life-history traits (2, 3). In mosquito vectors of human pathogens, for example, it has been well documented that conditions such as temperature (4–7), diet (8–11), competition (12–15), soil substrate (16, 17), and predator exposure (18) experienced during larval development can carry over and affect adult traits related to vectorial capacity. Vectorial capacity is a measure of vector-borne pathogen transmission potential that encapsulates the dynamics of vector-pathogen and vector-vertebrate host interactions (19), including vector life span and vector competence (that is, the intrinsic ability to acquire and subsequently transmit a pathogen).

Host-associated microbes, collectively known as the host microbiota, have manifold effects on host biology. Like other animals, insects establish symbiotic relationships with microbial communities that shape their physiological functions (20, 21). In recent years, it has become clear that the symbiotic microbiota of insect vectors play an important role in their vectorial capacity (20, 22). The native bacterial microbiota of

mosquitoes can modulate their immune response and vector competence for human pathogens (23–26). The relationship between mosquitoes and the endosymbiotic bacteria *Wolbachia* has been well documented, but the interactions between mosquitoes and their gut bacterial microbiota have not been described in such depth. Furthermore, our current understanding of how bacteria–insect vector interactions affect pathogen transmission is limited to adults. Little is known about whether the bacterial microbiota of larvae affect adult traits related to pathogen transmission. Our knowledge of bacterial communities in larval sites and between life stages is mainly descriptive (27–30), although it was recently shown that mosquito larvae rely on bacteria to develop (31–33).

Because the mosquito gut microbiota composition is dynamic and susceptible to environmental changes (20, 34–36), we hypothesized that habitat-related differences in bacterial communities in larval development sites could mediate environmental variation in vector-borne pathogen transmission. We addressed this question in the mosquito *Aedes aegypti*, an important worldwide vector of medically significant arboviruses such as dengue, Zika, yellow fever, and chikungunya viruses. In Sub-Saharan Africa, *A. aegypti* exist in the form of two ecotypes: a “sylvatic” ecotype of *A. aegypti* found in forested habitats, ecologically similar to the ancestral form of the species, and a human-adapted “domestic” ecotype that thrives in urbanized environments (37, 38). Whereas domestic *A. aegypti* larvae develop in artificial containers (cans, tires, jars, and flower pots) within or in close proximity to human habitation, the larvae of the sylvatic ecotype are typically found in natural breeding sites (rock pools, tree holes, and fruit husks).

First, we characterized the differences in the bacterial community composition between domestic and sylvatic *A. aegypti* larval development sites in Gabon and in the midguts of *A. aegypti* emerging from these sites. This initial description was used to justify our hypothesis that these differences may be functionally relevant at the adult stage. Second, we measured the variation in several adult traits related to vectorial capacity using gnotobiotic larvae (that is, sterile larvae subsequently exposed to a single bacterial isolate) made with native bacterial isolates

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from the same breeding sites in Gabon. In contrast with previous studies on bacteria-mosquito interactions that focused on the effect of a single bacterial isolate at the adult stage, our primary interest was to determine whether different bacteria-mosquito interactions at the larval stage could explain natural variation in adult traits. Dissecting the relative contribution of genetic and environmental factors in natural phenotypic variation is central to understand the evolutionary potential and mechanistic basis of a trait. Our study provides the proof of concept that exposure to different bacterial isolates during larval development results in variation in pupation rate and several adult phenotypes such as life-history traits and antimicrobial phenotypes.

## RESULTS

### Bacterial communities differ between domestic and sylvatic larval breeding sites

We compared the bacterial communities between domestic and sylvatic larval development sites and the midguts dissected from surface-sterilized adult *A. aegypti* females using a metagenomics approach based on targeted sequencing of the 16S ribosomal RNA gene (Fig. 1; for details, see Materials and Methods). Sylvatic samples were collected in gallery forests inside Lopé National Park, central Gabon, whereas domestic samples were collected in a nearby village. In the sylvatic environment, we characterized the bacterial communities in water collected from eight different *A. aegypti* larval development sites, nine midguts from adult females emerging from these sites, and six midguts from adult host-seeking females caught by human-landing catch (HLC) next to the larval development sites in the gallery forests. In the domestic environment, we characterized the bacterial communities in water collected from six *A. aegypti* larval development sites, eight midguts from adult females emerging from these sites, and three midguts from adult host-seeking females caught by HLC in the village. Overall, we analyzed eight water samples and 15 midguts from the sylvatic environment and six water samples and 11 midguts from the domestic environment. To control for contamination of bacteria introduced during sample processing, aliquots of reagents and blank samples were included as negative controls (for details, see Materials and Methods). A total of 2851 operational taxonomic units (OTUs) were identified among all the samples, of which 2412 were included in the analysis after removing OTUs that were present in the negative controls (that is, likely resulting from laboratory contamination).

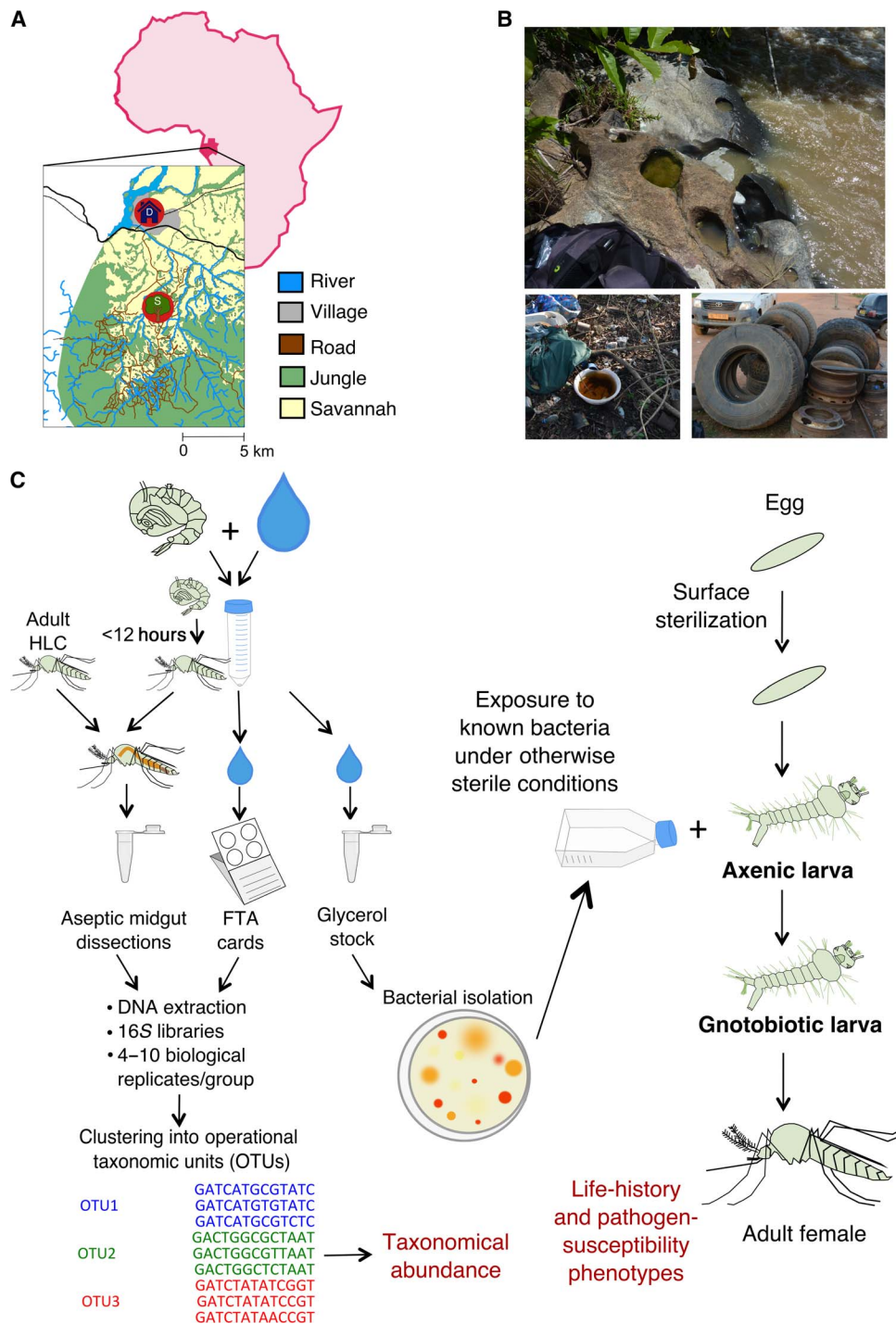
OTU richness was higher in the water samples than in the midgut samples, and it was higher in sylvatic water samples than in the domestic water samples (fig. S1A and file S1). Despite differences in the total number of distinct OTUs between sample type and habitat, there was no significant difference in the Shannon diversity index (fig. S1B and file S1). Note that because of the small sample sizes, the analyses of bacterial diversity were likely underpowered to draw any conclusions about the differences between the midguts from freshly emerged adults and those from HLC adults. Plotting the overlap of OTUs between sample types revealed that although some bacterial community members were shared, a large proportion was unique to each sample type (Fig. 2 and fig. S2). Within both the domestic and sylvatic habitats, there was only partial overlap between bacterial communities found in the midguts dissected following adult emergence, the midguts of HLC adults exposed to the natural environment, and water of larval development sites (Fig. 2, A and B). Notably, 28 OTUs (51%) found in the midguts from both freshly emerged and HLC adults were undetectable in the corresponding water samples.

This limited overlap between midgut and water samples was not simply due to differences in rare OTUs. Among the 100 most abundant OTUs, six OTUs that were abundant in midgut samples were undetected in the water they emerged from (fig. S3). Reciprocally, most of the OTUs that were abundant in water samples were not found in the midguts of adult emerging from them (fig. S3). The midguts from freshly emerged adults harbored a larger number of unique OTUs and, therefore, a larger number of OTUs overall, compared to those from HLC adults (Fig. 2, A and B). Approximately a third of the OTUs identified in the midguts following emergence were shared between domestic and sylvatic habitats (Fig. 2C). While more than half of the OTUs found in sylvatic water sites were unique to this habitat, a majority of OTUs found in domestic water sites overlapped with OTUs found in sylvatic water sites (Fig. 2D). Among the OTUs that were shared between sample types, the abundance of 137, 2, 495, and 291 OTUs differed significantly (Wald test) between domestic and sylvatic water samples, domestic and sylvatic midguts, sylvatic midguts and water samples, and domestic midguts and water samples, respectively (file S2).

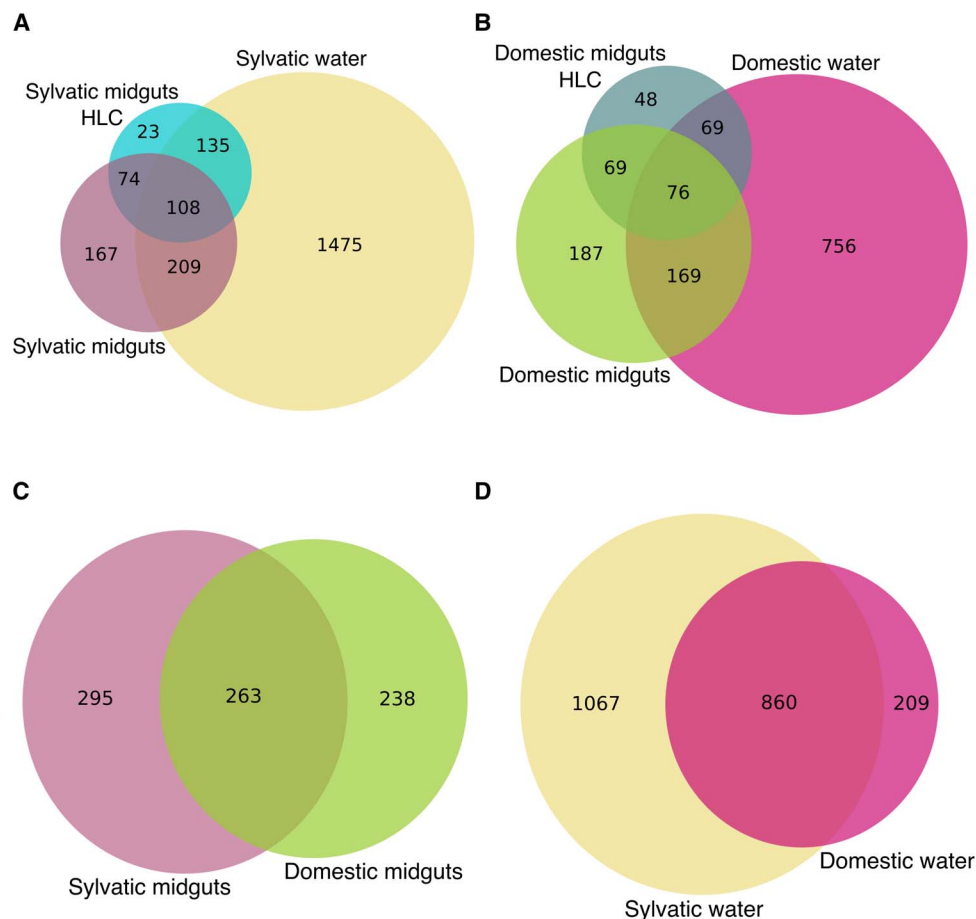
To determine whether the structure of bacterial communities differed between sample type and habitat, we used two different, complementary approaches (see Materials and Methods for details). In the first approach, a Bray-Curtis dissimilarity matrix was generated on the basis of OTU abundance and analyzed using nonmetric multidimensional scaling (Fig. 3A). In the second approach, a Bray-Curtis dissimilarity matrix was generated on the basis of *k*-mer presence/absence and analyzed by hierarchical clustering (Fig. 3B). Regardless of the approach, the structure of bacterial communities markedly differed ( $P = 0.001$ ) between water and midgut samples (Fig. 3). In addition, the structure of bacterial communities was distinct between domestic and sylvatic larval habitats (Fig. 3). Analyses of  $\beta$  diversity based on OTU counts confirmed that bacterial communities significantly differed between combinations of habitat and sample type ( $P = 0.005$ ). Lack of significant dispersion effects within habitat ( $P = 0.372$ ) and sample ( $P = 0.652$ ) types confirmed the validity of the statistical model. Without replicate water samples from the same larval breeding site, the degree of within-habitat heterogeneity could not be accurately quantified. However, one of the domestic water samples clustered with sylvatic water samples, pointing to some degree of heterogeneity among domestic sites (Fig. 3B). As noted above, because of the small sample sizes, these analyses were likely underpowered to draw any conclusions about the differences between the midguts from freshly emerged adults and those from HLC adults.

### Adult life-history traits vary between gnotobiotic larvae exposed to different bacterial isolates

To assess the functional relevance of differences in bacterial communities in larval development sites, we generated gnotobiotic *A. aegypti* larvae by exposing axenic (that is, bacteria-free) larvae to a single bacterial isolate during their development (Fig. 1; see Materials and Methods for details). We collected 168 bacterial isolates from the same larval development sites in Gabon in which we collected water for 16S targeted metagenomics. Of the 168 bacterial isolates, three were arbitrarily chosen for functional assays based on genetic dissimilarity, differences in the pupation rate of gnotobiotic larvae, and differences in the identity and proportion of midgut bacteria in adults emerging from gnotobiotic larvae (see Materials and Methods for complete description of the isolate screen). Two of the three isolates were isolated from domestic breeding sites, and on the basis of their full-length 16S sequence, they



**Fig. 1. Experimental workflow.** (A) Water and *A. aegypti* pupae (for later midgut dissection from emerging adults) were collected from sylvatic larval sites in gallery forests along the rivers and streams of Lopé National Park in Gabon and from domestic larval sites in a nearby village. (B) At domestic sites (lower pictures), samples were collected from artificial containers such as discarded plastic containers, tires, and metal tins. At sylvatic sites, samples were collected from rock pools (upper picture). (C) At each collection site, both water and pupae were collected into a sterile tube using a sterile pipette. The samples were brought back to the field station, and an aliquot of water was removed next to a Bunsen burner flame and frozen until processing. Back in the laboratory, the water samples were thawed and centrifuged, and the bacteria pellet was resuspended in sterile water and spotted on Whatman FTA cards for later DNA extraction. The pupae were held in the same collection tube until adults emerged. Midguts from adults were dissected within 12 hours of emergence next to a Bunsen burner flame and preserved for later DNA extraction. Midguts were also dissected from wild adult females caught by HLC. Deep-sequencing libraries were made using the V5-V6 hypervariable region of the 16S bacterial ribosomal RNA gene. The sequences were clustered into OTUs and used for analysis of taxonomical abundance and community structure. At the same time that an aliquot of water was frozen, another aliquot was also removed to make a glycerol stock. Upon return to the laboratory, the glycerol stocks were streaked out onto different medium types and individual colonies isolated. For functional assays *in vivo*, gnotobiotic larvae were created by adding a single bacterial isolate to sterile flasks containing axenic larvae. Adult mosquitoes that had undergone different gnotobiotic treatments as larvae were used to test for variation in life-history and antimicrobial phenotypes.

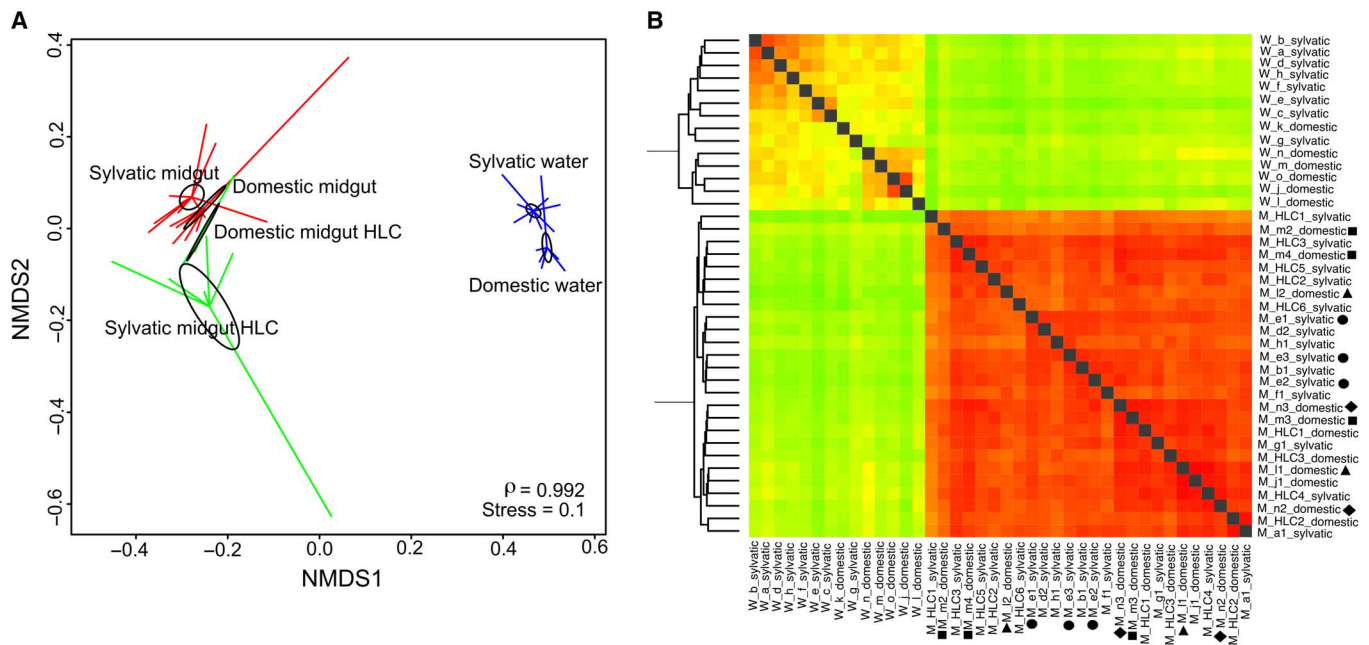


**Fig. 2. Limited overlap of bacterial communities between habitat and sample types.** Venn diagrams show the overlap between OTUs identified in (A) samples from the sylvatic environment, (B) samples from the domestic environment, (C) mosquito midguts, or (D) water samples. OTU diversity indices, taxonomical composition, and relative abundance by sample type are shown in figs. S1, S2, and S3, respectively. The normalized OTU count table is provided in file S8.

were assigned to *Salmonella* (Ssp\_ivi) and *Rhizobium* (Rsp\_ivi) genera. The third isolate (Esp\_ivi) was isolated from a sylvatic breeding site and belongs to the Enterobacteriaceae family; however, classification at the genus level was inconsistent among databases (alternatively *Salmonella*, *Escherichia*, or *Shigella*). In the 16S data set, the Enterobacteriaceae and *Rhizobium* taxonomical groups were present in both domestic and sylvatic breeding sites, whereas the *Salmonella* taxonomical group was only found in domestic breeding sites. Note that the isolates were not chosen to reflect the dominant taxa identified by the targeted metagenomics approach. To minimize the potential confounding effects of specific interactions between mosquito genotypes and sympatric bacterial isolates, gnotobiotic mosquitoes were created using a wild-type mosquito genetic background from Thailand. We measured pupation rates in gnotobiotic larvae to determine whether the interaction with different bacteria present in the water alters larval development. As previously reported (31, 32), when larvae were maintained as axenic, the larvae did not develop past the first instar stage (Fig. 4A). To assess the differences in the pupation rate of the different gnotobiotic treatments, we compared the growth rate (that is, slope of the exponential phase) and the time it took for 50% of the larvae to pupate using a three-parameter model of pupation dynamics (Fig. 4A). The nonaxenic larvae had a significantly faster growth rate than the gnotobiotic larvae, but no significant differences in growth rate were observed among the gnotobiotic larvae (file S3). This is in contrast to the initial screen of bacterial

isolates, which is likely a result of greater statistical power in this data set; the initial screen consisted of three replicate flasks of larvae per treatment, whereas this data set included three replicate flasks per treatment from three independent experiments. Although the larval growth rate during the exponential phase was similar among gnotobiotic treatments, the time it took to reach 50% pupation significantly differed among treatments (file S3). The lag phase was shorter for larvae exposed to the Ssp\_ivi isolate than for those exposed to the Esp\_ivi or Rsp\_ivi isolate, whereas there was no difference between larvae exposed to the Esp\_ivi or Rsp\_ivi isolate (Fig. 4A). The pupation rate at day 5, 9, or 12 of larval development was not dependent on the amount of bacteria in the flask on the corresponding day, nor was the pupation rate at day 9 dependent on the amount of bacteria inoculated into individual flasks upon egg hatching (file S4).

To assess the fitness of adult mosquitoes after being exposed to different bacterial isolates during larval development, we measured their life span and wing length (a proxy for body size). The life span of adult females did not significantly differ ( $P = 0.593$ ) between gnotobiotic treatments (Fig. 4B), but there were significant differences ( $P = 8.6 \times 10^{-6}$ ) in their wing length (Fig. 4C and Table 1). The larvae that were exposed to the Ssp\_ivi isolate grew into adults with the largest wings, the Esp\_ivi and nonaxenic treatments resulted in the smallest wing length, and the Rsp\_ivi treatment resulted in an intermediate wing length.



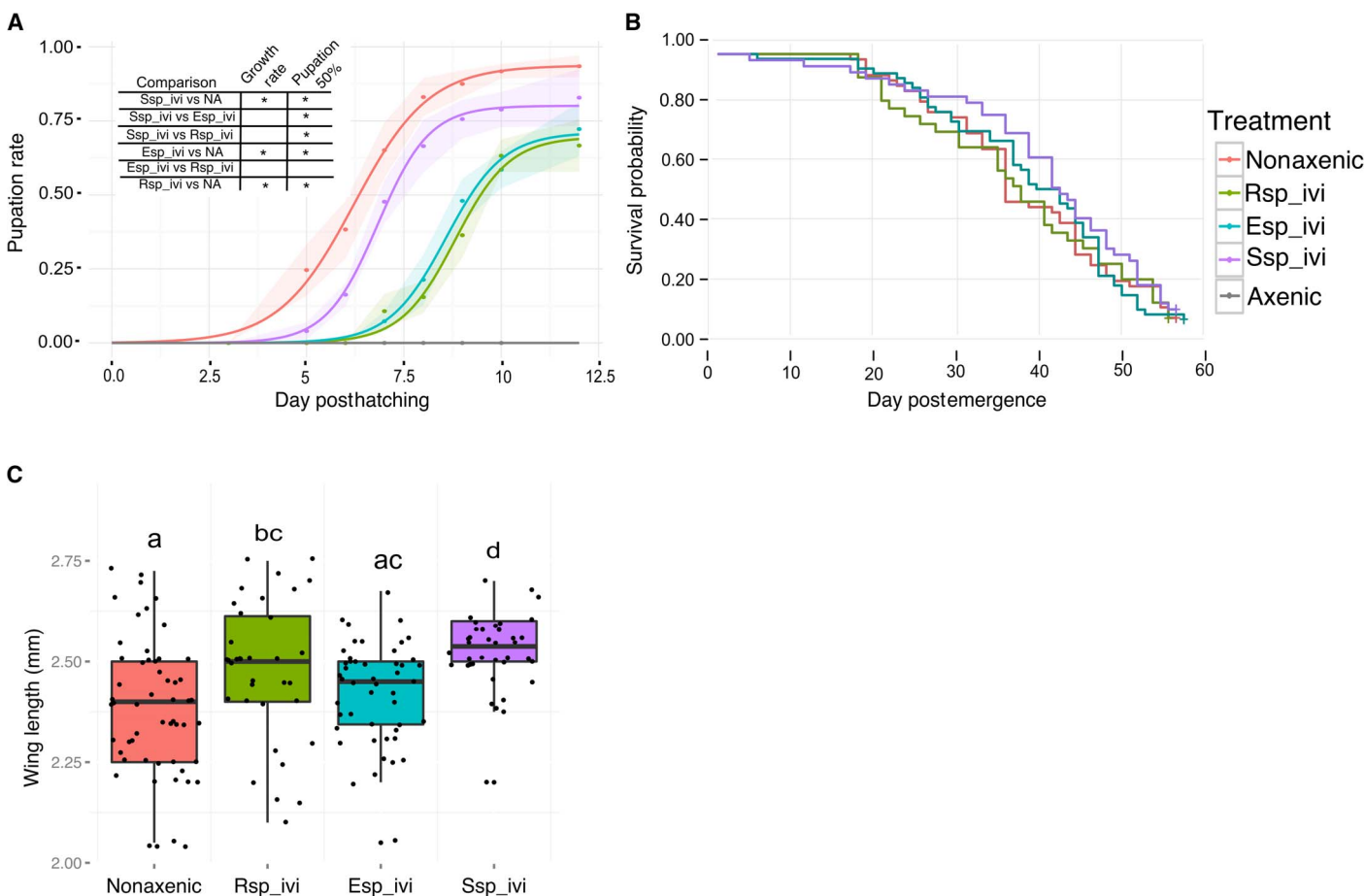
**Fig. 3. Bacterial community structures differ between domestic larval development sites, sylvatic larval development sites, and mosquito midguts.** Structure of bacterial communities was determined by deep sequencing the V5-V6 region of the bacterial 16S gene among individual samples and sample types, including domestic and sylvatic water samples, domestic and sylvatic midguts, and midguts dissected following emergence and collected by HLC. Bacterial community structure is represented by (A) nonmetric multidimensional scaling (NMDS) of Bray-Curtis dissimilarity index based on OTU abundance and (B) heat map of Bray-Curtis dissimilarity index based on  $k$ -mer presence/absence and hierarchical clustering. In the NMDS plot, Spearman correlation ( $\rho$ ) and stress values are indicated. The normalized OTU count table used to perform the NMDS analysis is provided in file S8. In the heat map, samples are labeled according to their type (M, midgut; W, water) and habitat of origin (sylvatic or domestic). Midguts dissected following emergence are labeled to match the corresponding water sample (for example, midgut a1 was dissected from a mosquito that emerged from water sample a). Midguts from the same breeding site are marked with matching symbols. Red color indicates high similarity, and green color indicates low similarity.

### Adult antimicrobial phenotypes vary between gnotobiotic larvae exposed to different bacterial isolates

To determine whether exposure to different bacterial isolates during larval development results in variation in susceptibility to microbes as adults, we measured the antibacterial activity of the hemolymph and the susceptibility to dengue virus of adult females emerging from gnotobiotic larvae. In mosquitoes, the hemolymph displays a strong immune response and is involved in immune priming and immune memory (39–41). To test for differences in the immune system of adult *A. aegypti* that had been exposed to different bacteria during larval development, we measured the antibacterial activity of the hemolymph based on its ability to clear *Micrococcus luteus* on an agar plate (see Materials and Methods for details), as previously described (42, 43). In individual mosquitoes whose hemolymph resulted in detectable clearance of *M. luteus*, there was no significant difference ( $P = 0.207$ ) in the intensity of *M. luteus* clearance among the three gnotobiotic treatments. However, there was a significant difference ( $P = 0.025$ ) in the proportion of individuals whose hemolymph demonstrated detectable *M. luteus* clearance. Hemolymph collected from adult females who had been exposed to the Esp\_ivi isolate as larvae showed significantly fewer individuals who were able to clear *M. luteus* (Fig. 5A and Table 1).

Next, we examined whether the effect of Esp\_ivi exposure during larval development only affected adult antibacterial immunity or was also involved in carryover effects on susceptibility to virus infection at the adult stage. We measured the variation in susceptibility to dengue virus (serotype 1) in adult *A. aegypti* females who had been exposed to

different bacterial isolates during larval development. In two separate experiments, we measured the proportion of mosquitoes that became infected, the proportion of infected mosquitoes that developed a disseminated (that is, systemic) dengue virus infection, and the infectious titer of disseminated dengue virus in the head tissues 14 days after an infectious blood meal. Infection prevalence could be analyzed only in the first experiment because 94.5% of mosquitoes became infected in the second experiment. In the first experiment, 63.2% of mosquitoes were infected overall, and infection prevalence was not significantly affected by the gnotobiotic treatment ( $P = 0.3256$ ; fig. S4). For the same reason as above, we only analyzed the dissemination prevalence in the second experiment because 95.8% of infected mosquitoes had a disseminated infection in the first experiment. Differences in infection and dissemination rates were the result of different infectious doses used in the two experiments (see Materials and Methods for details). In the second experiment, 70.2% of mosquitoes had a disseminated infection overall, and dissemination prevalence was not significantly affected by the gnotobiotic treatment ( $P = 0.8579$ ; fig. S4). Among mosquitoes with a disseminated virus infection, we found modest but statistically significant differences in the infectious titer measured in the head tissues of adults exposed to the Esp\_ivi and Ssp\_ivi isolates as larvae (Table 1). Specifically, females exposed to the Esp\_ivi isolate during larval development had fewer viral particles in the head than those exposed to Ssp\_ivi (Fig. 5B). Lack of a significant experiment-by-isolate interaction with regard to virus titer indicated that the effect of the isolate on virus titer was consistent across both experiments (Table 1).



**Fig. 4. Different larval gnotobiotic treatments result in variation in pupation rate and adult body size, but not in adult life span.** (A) Pupation rate was determined by counting the number of pupae each day in three replicate flasks of gnotobiotic and nonaxenic larvae in three independent experiments. Axenic larvae (gray line) were included as negative controls. Statistical significance of pairwise differences in pupation rate between treatments was determined by using a three-parameter model to compare the slope of the exponential phase and the day when 50% of larvae pupated. Statistical significance for each pairwise comparison is indicated by a star in the inset table. The shaded ribbon around each curve represents SEM. (B) Adult female life span was determined by counting the number of dead females in triplicate cages. No statistical difference in life span was detected between the different treatments ( $P = 0.54$ ). (C) Boxplots represent the wing length of adult females from different gnotobiotic treatments. Statistical significance of pairwise differences between treatments was determined by  $t$  test. Letters above the graph indicate statistical significance in which treatments with a letter in common are not significantly different from each other.

## DISCUSSION

To the best of our knowledge, we provide the first empirical evidence that exposure to different bacteria during larval development can result in variation in adult traits related to pathogen transmission by an important insect vector. We observed differences in the bacterial communities that inhabit ecologically distinct *A. aegypti* breeding sites in Gabon. Using native bacterial isolates derived from these natural breeding sites, we created gnotobiotic mosquitoes to reveal the functional consequences of differential bacterial exposure at the larval stage. These results improve our understanding of environmentally mediated effects that carry over from one life stage to another life stage in holometabolous insects. They also emphasize the importance of accounting for larval ecology to unravel the determinants of pathogen transmission by insect vectors of human pathogens.

The first aim of our study was to describe habitat-related differences in the bacterial composition of *A. aegypti* larval development sites. Our observation of distinct bacterial communities between domestic and sylvatic habitats supports previous observations of habitat-related differences in bacterial communities in mosquito larval sites (27–30)

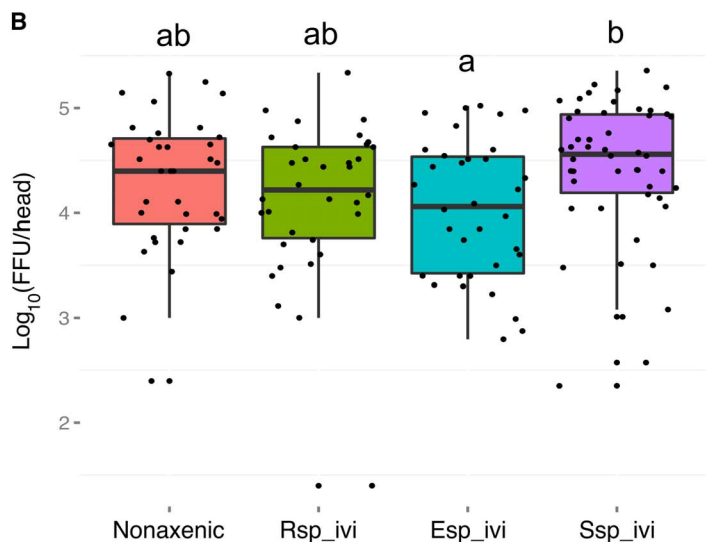
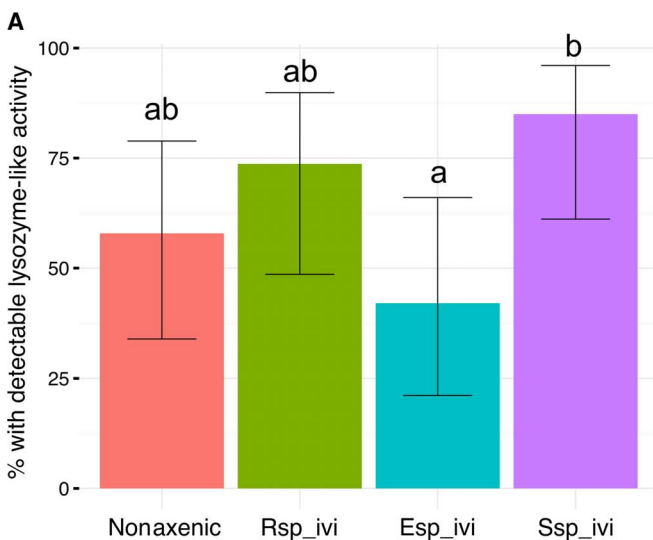
and verifies an important assumption of our study. Another marked observation from our targeted metagenomics approach was the dissimilarity between bacteria found in the larval site water and those in the adult midguts emerging from these same sites. The bacterial composition is known to vary between the larval and adult mosquito midguts (44–49), and adult mosquitoes are thought to have lost most of their midgut bacteria during metamorphosis (50, 51). However, recent work has also shown that several bacterial community members in *A. aegypti* are transstadially transmitted (32) and that adult *Anopheles* may acquire their gut community from larval breeding sites (52). The limited overlap in bacterial OTUs between the matched water samples and the midguts from freshly emerged adults could be explained by at least five scenarios. (i) The OTUs that we detected in the adult midguts were also present in the water but at a frequency that was too low to be detected by our sequencing method. (ii) The bacteria found in the adult midguts were not acquired from the water during larval development and, instead, were inherited in alternative ways such as vertical transmission. *Wolbachia* can be inherited vertically in *Culex* species and *Aedes albopictus* (20, 53, 54), and vertical transmission of the bacterium *Asaia* has been observed in

**Table 1. Test statistics of wing length, hemolymph lysozyme-like activity, and titer of disseminated dengue virus.** Wing length was compared with an analysis of variance. The proportion of hemolymph extracts with detectable antibacterial activity were analyzed with a logistic regression and analysis of deviance. FFU counts in head tissues were  $\log_{10}$ -transformed and compared with an analysis of variance. With the exception of wing length, the model includes the effect of the isolate (Ssp\_ivi, Esp\_ivi, Rsp\_ivi, and nonaxenic), the experiment (two repetitions), and their interaction. Df, degrees of freedom; LR, likelihood ratio. \* $P < 0.05$ ; \*\* $P < 0.01$ ; \*\*\* $P < 0.001$ .

Wing length			
	Df	F	P
Isolate	3	9.415	$8.635 \times 10^{-6}$ ***
Lysozyme-like activity in adult hemolymph			
	Df	LR $\chi^2$	P
Isolate	3	9.343	0.0251 *
Experiment	1	2.150	0.1426
Isolate $\times$ experiment	3	3.272	0.3516
Dengue virus FFU in adult head tissues			
	Df	F	P
Isolate	3	2.716	0.0470 *
Experiment	1	0.211	0.6471
Isolate $\times$ experiment	3	1.434	0.2355

*Anopheles* mosquitoes (55). (iii) The bacteria found in the adult midguts were not present in the water but were acquired during the larval stage from larvae feeding on organic detritus such as leaves, wood, or other arthropods. (iv) The bacteria identified in the midguts were acquired from a different water depth than we sequenced. We did not control for the depth of the water that we collected for sequencing. It is possible that bacterial communities differ between water at the bottom and at the top of a pool (56) and that the bacteria that we identified in the mosquito midguts were acquired from the bottom of the pools or vice versa. (v) The low sample size of eight to nine midguts from freshly emerged adults per habitat may not capture the true taxonomical diversity. There is great variability in the composition of mosquito midgut bacterial microbiota, and we may need a larger sample size to capture all taxa present. Finally, we found that the number of OTUs detected in the midguts (that is, OTU richness) was significantly smaller than that in the water samples. This finding is consistent with previous reports of the low complexity of bacterial communities typically found in mosquito midguts (20, 57).

The second aim of this study was to assess whether exposure to different bacteria during larval development resulted in changes in adult traits involved in vectorial capacity. It was previously observed that the addition of any bacteria to axenic mosquito larvae rescued their development and allowed them to pupate and become adults (31, 32). Although the authors of the study did not elucidate the mechanism underlying this observation, they hypothesized that the requirement for bacteria in the larval water was not nutritional because nonaxenic larvae maintained on sterile food in a sterile environment were still able to develop. Our observation of differences in pupation rate and adult body size hints at a possible role of nutrition and/or metabolism and deserves further work. Despite differences in pupation rate and adult size, the life span of adults exposed to different bacteria as larvae was



**Fig. 5. Adult antimicrobial phenotypes vary following different larval gnotobiotic treatments.** (A) Antibacterial activity of the hemolymph. Bars show the percentage of individual adult mosquitoes whose hemolymph was able to inhibit *M. luteus* growth on an agar plate in two replicate experiments of 10 females each. Vertical bars indicate 95% confidence intervals of the percentages. Statistical significance of pairwise differences was determined with a  $\chi^2$  test. (B) Infectious titer of dengue virus disseminated to the head tissues. The boxplot represents the concentration of infectious dengue virus particles expressed as the  $\log_{10}$ -transformed number of focus-forming units (FFU) in the head of adult females 14 days after oral exposure. Statistical significance of pairwise differences was determined with a *t* test. Prevalence of midgut infection and systemic viral dissemination in both experiments is shown in fig. S4. Letters above the graphs indicate statistical significance in which treatments with a letter in common are not significantly different from each other.

similar regardless of the bacterial isolate. It is possible that other bacteria besides the three isolates that we used could alter adult life span, as was shown for the endosymbiont *Wolbachia* in *Aedes* mosquitoes (58, 59).

We observed differences in adult susceptibility to systemic dengue virus dissemination and differences in the innate immune response to *M. luteus*. Mosquito hemolymph is regarded as an essential component of the immune system and plays an important role in pathogen recognition and elimination and in immune memory. We detected differences in the ability of hemolymph collected from adults exposed to different bacteria as larvae to clear *M. luteus* on an agar plate. Specifically, hemolymph from adults exposed to the Esp\_ivi isolate during larval development was less efficient at clearing *M. luteus* compared to other gnotobiotic treatments. Protection against *M. luteus* does not imply uniform protection across all bacteria species, and further work remains to be carried out to determine whether the effect we observed extends to other types of bacteria. It was the Esp\_ivi treatment that resulted in adults who were better at controlling systemic dengue virus dissemination (that is, less infectious viral particles in the head tissues), pointing to a potential trade-off between bacterial defense and the ability to control viral infections. Infectious titer of disseminated dengue virus is positively correlated with the probability of virus detection in *A. aegypti* saliva (60) and is often used as a proxy for transmission potential. Adult body size has been shown to influence the susceptibility of *A. aegypti* to viral infection (61), but differences in wing length did not explain the differences observed in dengue virus dissemination in our experiments. Both the nonaxenic and Esp\_ivi treatments resulted in significantly smaller adults; however, the level of viral dissemination was high in the control and low in the Esp\_ivi treatment.

Differences in hemolymph antibacterial activity and titer of disseminated dengue virus, despite similar midgut infection rates the gnotobiotic treatments, are consistent with those in hemolymph-mediated immune priming. Note that although our results suggest immune differences among different gnotobiotic treatments, further work will be necessary to establish a link between specific immune mechanisms and susceptibility to bacterial colonization of the midgut and dengue virus dissemination. An alternative explanation is that differences in bacterial exposure during larval development result in differences in the composition of the gut bacterial microbiota of adult mosquitoes, which could indirectly modulate the antibacterial and antiviral immune responses. In line with this hypothesis, we observed differences in the bacterial composition and the number of cultivable bacteria present in the midguts of adults who underwent different gnotobiotic treatments (file S3), although there was no clear correlation between different bacterial communities and the phenotypes tested.

Together, our results provide the proof of principle that exposure to different bacteria during larval development can influence the variation in adult traits in the holometabolous insect *A. aegypti*. By building on the observation that in Gabon, the composition of bacterial communities differs between ecologically distinct *A. aegypti* larval development sites, we demonstrated that experimental exposure to different natural bacterial isolates at the larval stage can influence the potential transmission of a medically relevant human pathogen. *A. aegypti* larvae in nature would not be exposed to a single bacterium like in our gnotobiotic experiments, which did not fully capture the true complexity of a natural situation. Because of the inability to culture all bacteria present in larval development sites and the logistical difficulties to recreate relevant natural bacterial communities under laboratory settings, our study rather establishes the proof of concept that habitat-mediated differences in the bacterial communities of *A. aegypti* larval sites can influ-

ence adult traits. Evidence that differential larval exposure to bacteria, and thus larval ecology, may contribute to phenotypic variation in mosquito vectorial capacity is an important step toward a more comprehensive understanding of how environmental conditions shape the risk of vector-borne disease.

## MATERIALS AND METHODS

### Ethical statement

The Institut Pasteur animal facility received accreditation from the French Ministry of Agriculture to perform experiments on live animals in compliance with the French and European regulations on the care and protection of laboratory animals. Rabbit blood draws performed in the context of this study were approved by the Institutional Animal Care and Use Committee at Institut Pasteur under protocol number 2015-0032. Mosquito collections inside Lopé National Park were conducted under permit number AR0020/14/MESR/CENAREST/CG/CST/CSAR. Mosquito collections by HLC in Gabon were performed under protocol number 0031/2014/SG/CNE approved by the National Research Ethics Committee.

### Field sampling

Water from larval breeding sites and midguts of *A. aegypti* females emerging from the same larval sites were collected in Gabon in November 2014. Sylvatic collections were made inside Lopé National Park (latitude,  $-0.148617$ ; longitude,  $11.616715$ ), and domestic collections were made in Lopé village (latitude,  $-0.099221$ ; longitude,  $11.600330$ ) approximately 6 km from the sylvatic collection sites (Fig. 1). All of the sylvatic collections originated from rock pools, and the domestic collections were from various types of artificial containers and tires (Fig. 1B and file S5). At each larval breeding site, pupae and water were collected into a sterile 50-ml conical tube with filter-top lid using a sterile plastic pipette and brought back to the Station d'Etude des Gorilles et Chimpanzés field station. Upon arrival at the field station, 10 ml of water was transferred to a new sterile 50-ml conical tube next to the flame of a Bunsen burner and immediately stored at  $-20^{\circ}\text{C}$ . The remaining water and pupae were held until the adults emerged and were visually identified as *A. aegypti*. The frozen water was transported back to the Centre International de Recherches Médicales de Franceville facilities in Franceville, Gabon, where the water was thawed and centrifuged at 3400 rpm for 10 min. Under a laminar flow cabinet, the supernatant was removed and replaced with 500  $\mu\text{l}$  of sterile water to resuspend the bacterial pellet. The resuspended bacteria were spotted onto Whatman FTA cards (WB120401, GE Life Sciences), allowed to dry, and then wrapped in sterile foil for transport to Institut Pasteur in Paris.

Within 12 hours of adult emergence, the midguts from *A. aegypti* females were dissected and stored in RNAlater (Qiagen) at  $+4^{\circ}\text{C}$ . RNAlater was initially chosen to preserve the midgut tissue with the hope of being able to recover both RNA and DNA from the samples, but it was not possible to isolate a sufficient amount of RNA and DNA from each sample, so only DNA extractions were performed. Because of the lack of a laminar flow cabinet at the field station in Lopé National Park, the midguts were dissected within 50 cm of a Bunsen burner flame in an effort to maintain sterility. Before and between each dissection, the dissecting tools were disinfected with 3% bleach. Adult *A. aegypti* were removed from the tube in which they emerged and were cold-anesthetized. The mosquito was then surface-sterilized in 3% bleach and rinsed in sterile phosphate-buffered saline (PBS), and the midgut was dissected in a drop of sterile PBS. Because of the limited access to



ethanol in the field, surface sterilization was only performed with 3% bleach. Negative controls were included in an attempt to control for potential contamination of bacteria introduced at this step (see below). The dissected midguts were placed in individual sterile tubes containing RNAlater that had been filtered through a 0.2- $\mu$ m filter and aliquoted under sterile conditions. The midguts in RNAlater were stored at +4°C until being frozen at –20°C upon their arrival at Institut Pasteur in Paris until the DNA extraction was performed. Following the same procedure, the midguts from non–blood-fed, host-seeking adult females caught by HLC were also dissected. Human volunteers sat next to either the rock pools (sylvatic habitat) or the artificial containers (domestic habitat) where *A. aegypti* had been previously observed and caught the females as they landed and were preparing to probe.

### DNA extractions

DNA from bacteria originating from the water samples was extracted from the Whatman FTA cards following the organic DNA extraction procedure provided by Whatman. DNA extractions were performed on two consecutive days, mixing samples each day to randomize a potential batch effect. Briefly, the filter paper was cut into small pieces using sterile scissors and soaked in 500  $\mu$ l of extraction buffer [10 mM tris-HCl (pH 8.0), 10 mM EDTA, disodium salt (pH 8.0), 100 mM sodium chloride, and 2% (v/v) SDS] and 20  $\mu$ l of proteinase K (20 mg/ml) prepared in sterile water overnight at 56°C with agitation. An equal volume of buffered phenol (pH 8.0) was added, vortexed briefly, and centrifuged for 10 min at maximum speed. The upper aqueous phase was transferred to a new microcentrifuge tube containing 500  $\mu$ l of chloroform, vortexed thoroughly, and centrifuged for 10 min at maximum speed. The upper aqueous phase was transferred to a new tube containing 50  $\mu$ l of 3 M sodium acetate (pH 5.2). Eight hundred microliters of 100% ethanol was added and vortexed. The DNA was allowed to precipitate at –20°C for at least 1.5 hours. The DNA was recovered by centrifuging for 30 min at maximum speed. The supernatant was discarded, and 1 ml of 70% ethanol was added to the pellet and centrifuged for 20 min at maximum speed. The supernatant was removed, and the pellet was air-dried for 30 min and dissolved in 50  $\mu$ l of sterile water. Because of the large number of samples, the extractions were performed in two batches. To control for contamination of bacteria introduced during the DNA extraction, a negative control was made from each day of extraction by performing the extraction on a blank sample.

Upon thawing the samples, the midguts were removed from the RNAlater and added to 300  $\mu$ l of lysozyme (20 mg/ml) dissolved in Qiagen ATL buffer in a sterile tube containing grinding beads. The samples were homogenized for two rounds of 30 s at 6700 rpm (Precellys 24, Bertin Technologies). The samples were incubated at 37°C for 2 hours, after which 20  $\mu$ l of proteinase K was added and vortexed briefly, followed by another incubation of 4 hours at 56°C on a shaker (300 rpm). After the incubation, 200  $\mu$ l of Qiagen AL buffer and 200  $\mu$ l of 100% ethanol were added to the samples and vortexed to mix. The lysate was transferred to a Qiagen DNeasy Mini Spin column (except midguts from HLC adults that were passed through Qiagen AllPrep columns), washed with buffers Qiagen AW1 and AW2 following kit instructions, and eluted in 20  $\mu$ l of sterile water. To control for contamination of bacteria introduced both during the midgut dissections in the field and during the DNA extraction in the laboratory, negative controls were made by performing the same DNA extraction procedure on an aliquot of RNAlater opened at the field station in Gabon, an aliquot of sterile PBS used for midgut dissections that was opened at the field station in Gabon, and a blank midgut sample.

### 16S sequencing

Libraries were made from 8 sylvatic water samples, 6 domestic water samples, 15 sylvatic midguts (9 freshly emerged and 6 HLC adults), and 11 domestic midguts (8 freshly emerged and 3 HLC adults). In addition, two technical replicates were prepared with different primer pairs used on the same water samples. Technical replicates were used to confirm the repeatability of the sequencing results. Custom-made polymerase chain reaction (PCR) primers targeting the hypervariable V5-V6 region of the bacterial 16S ribosomal RNA gene were designed following Fadrosch *et al.* (62). These custom primers were designed to include the necessary Illumina adapters and indexes so that only one round of PCR was needed and therefore avoid multiple rounds of PCR that could lead to a sampling bias. To overcome the issues that arise when sequencing libraries with low-diversity sequences, such as PCR amplicons, heterogeneity spacers consisting of 0 to 7 base pairs were added to the custom primers so that the sequences would be sequenced out of phase (62). A total of eight forward and eight reverse primers were designed (file S6) and used in all 8  $\times$  8 combinations to amplify all the breeding site water and midgut samples. Four microliters of each breeding site water sample and 6  $\mu$ l of each midgut sample were used to amplify the V5-V6 16S region in triplicate using Expand High-Fidelity polymerase (Sigma-Aldrich) following the manufacturer's instructions, with the addition 0.15  $\mu$ l of T4gene32 and 0.5  $\mu$ l of bovine serum albumin (20 mg/ml) per reaction to improve PCR sensitivity. Water samples were amplified for 30 cycles, and midgut samples were amplified for 40 cycles. The three PCRs were pooled, and the PCR products were purified using Agencourt AMPure XP magnetic beads (Beckman Coulter). The purified PCR products were quantified by Quant-iT PicoGreen dsDNA fluorometric quantification (Thermo Fisher Scientific) and pooled for sequencing on the Illumina MiSeq platform (Illumina). The sequencing run failed multiple times with no achievable explanation except for the inability of the sequences to bind to the flow cell. The failed custom sequencing tags were replaced with sequencing tags used successfully in previous projects (57), which required performing a second round of PCR because all extracted DNA from the midgut samples had been used in the initial PCR. The second round of PCR used new custom primers containing the same V5-V6 region to rescue the samples (file S7). One microliter of each library was amplified in triplicate using Expand High-Fidelity polymerase (Sigma-Aldrich) for eight PCR cycles. The three PCRs were pooled, purified using AMPure XP magnetic beads (Beckman Coulter), and quantified using Quant-iT PicoGreen dsDNA fluorometric quantification (Invitrogen). Library quality was checked by Bioanalyzer (Agilent Technologies), and 300–base pair paired-end sequences were generated on the Illumina MiSeq platform using a V3 kit (Illumina). The raw sequence data are available at the European Nucleotide Archive under accession number PRJEB16334.

### Targeted bacterial metagenomics analysis

Read filtering, OTU clustering, and annotation were performed with the MASQUE pipeline (<https://github.com/aghazlane/masque>), as described by Quereda *et al.* (63). A total of 2851 OTUs were obtained at 97% sequence identity threshold. The statistical analyses were performed with SHAMAN ([shaman.c3bi.pasteur.fr](http://shaman.c3bi.pasteur.fr)) based on R software (v3.1.1) and bioconductor packages (v2.14). Because bacterial communities were expected to differ substantially between mosquito midguts and water samples, the normalization of OTU counts was performed at

the OTU level by sample type (midgut or water) using the DESeq2 normalization method. All samples including the negative controls and technical replicates were included in the normalization step. The technical replicates were removed from the data set before analysis. To account for possible contamination at various steps in the sample-processing pipeline, the OTU counts were corrected with the reads from the negative controls (see above). All OTUs found in the negative control samples were removed from the normalized OTU table unless the count in a real sample was >10 times higher than the mean OTU count in the negative controls. This operation was performed with a homemade script in R (64). This normalized OTU count table with the OTUs found in the negative controls removed (file S8) was used for the richness, Shannon index, Venn diagrams, abundance heat map, and NMDS analysis. Observed richness, Shannon index, and Bray-Curtis distances were calculated with the vegan package in R (65). The effects of sample types and ecotypes on the bacterial richness were tested by fitting a generalized linear model (GLM) with a Poisson distribution. The SEs were corrected for overdispersion using a quasi-GLM model where the variance is given by the mean multiplied by the dispersion parameter. A  $\chi^2$  test was applied to compare the significance of deviance shift after adding the covariates sequentially. The effects of sample type, ecotype, and their interaction on Shannon index were tested by fitting a linear model with a normal error distribution. The response variable was power-transformed to satisfy the model assumptions. The significance of each variable was tested with an analysis of variance (ANOVA) after adding the covariates sequentially. The results of the two models were confirmed by the convergence of backward and forward selection based on the Akaike information criterion. The Bray-Curtis distances were plotted with an NMDS method constrained in two dimensions. The Spearman correlation with real distances and stress value was estimated with the vegan R package (65). Effects of habitat and sample type on  $\beta$  diversity were tested with the betadisper and adonis permutational multivariate ANOVA methods from the vegan R package with 999 permutations of the Bray-Curtis distance matrix derived from OTU counts.

In SHAMAN, a GLM was fitted and vectors of contrasts were defined to determine the significance in abundance variation between sample types. The GLM included the main effect of habitat (sylvatic or domestic), the main effect of sample type (midgut or water), and their interaction. The resulting *P* values were adjusted for multiple testing according to the Benjamini and Hochberg procedure. All OTUs that were present in the negative controls were excluded from the final list of differentially abundant OTUs.

To confirm the OTU-based results with an OTU-independent method, a dissimilarity matrix was generated with the SIMKA software (66). Reads with a positive match against the sequences assembled from the negative controls were removed using Bowtie v2.2.9 (67). Then, *k*-mers of size 32 and occurring at least greater than two times were identified with SIMKA. Bray-Curtis dissimilarity was estimated between each sample.

### Bacterial isolation

At the same time water was removed to freeze for DNA extraction, an aliquot of the larval site water was added to 50% sterile glycerol to make 20% glycerol stocks of the larval site water. The glycerol stocks were frozen at  $-20^{\circ}\text{C}$  until they were transported back to Institut Pasteur in Paris. Upon arrival in Paris, the glycerol stocks were streaked out onto agar plates made with LB medium [LBm; LB with NaCl (5 mg/ml)] and PYC

medium [peptone (5 g/liter), yeast extract (3 g/liter), and 6 mM calcium chloride dihydrate ( $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ ) (pH 7.0)] plates and incubated for 3 days at  $30^{\circ}\text{C}$ . LBm and PYC were chosen for being generalist media. Individual colonies were picked from the plates and used to inoculate 3 ml of the appropriate media, which were shaken at  $30^{\circ}\text{C}$  until bacterial growth occurred and used to create new glycerol stocks of the individual isolates. The same colony was also put into 20  $\mu\text{l}$  of sterile water and exposed to two rounds at  $95^{\circ}\text{C}$  for 2 min and resting on ice for 2 min. The samples were then centrifuged for 5 min at maximum speed to remove cell debris, and the supernatant was used to amplify the entire 16S region by PCR [5'-AGAGTTTGATCCTGGCTCAG-3' (forward) and 5'-AAGGAGGTGATCCAGCCGCA-3' (reverse)] using Expand High-Fidelity Polymerase (Sigma-Aldrich). The PCR products were purified using the QIAquick PCR Purification kit (Qiagen), quantified by NanoDrop (NanoDrop Technologies Inc.), and sequenced by Sanger sequencing. The sequences were aligned and classified at the genus level using the SILVA database ([www.arb-silva.de/](http://www.arb-silva.de/)). The raw sequence data are available at the European Nucleotide Archive under accession number PRJEB16334. Individual colonies were chosen on the basis of size, color, and morphology. The purity of the colonies used in the gnotobiotic experiments was verified by restreaking the bacteria on multiple occasions.

### Gnotobiotic larvae

Axenic larvae were created using the eighth generation of an *A. aegypti* laboratory colony derived from a natural population originally sampled in Thep Na Korn, Kamphaeng Phet Province, Thailand, in 2013. This mosquito strain was used to create gnotobiotic larvae as a common genetic background from a different geographical region that had, presumably, not encountered the specific bacterial isolates introduced. The rationale was to avoid potentially confounding effects of local adaptation between mosquitoes and bacterial isolates. Eggs were gently scraped off the paper they were laid on into a 50-ml conical tube. The eggs were incubated in 70% ethanol for 5 min, 3% bleach for 3 min, and 70% ethanol for 5 min. The eggs were then rinsed in sterile water three times and allowed to hatch in sterile water in a vacuum chamber. Upon hatching, the larvae were transferred to sterile 25-ml tissue-culture flasks with filter-top lids and maintained in 15 ml of sterile water. Larvae were seeded to a density of 10 to 15 larvae per flask. The larvae were maintained on 50  $\mu\text{l}$  of sterile fish food every other day. The water of the larval flask was not changed for the duration of the experiment. Fish food was made sterile by resuspending ground-up fish food with water and autoclaving it for 20 min. Axenic larvae were made gnotobiotic by adding a single bacterial isolate of choice. One to 3 days before inoculating the larval flasks, the bacteria were streaked out onto agar plates with their appropriate medium. They were allowed to grow 1 to 3 days until colonies of roughly similar size were obtained. A single bacterial colony was picked and added to each 25-ml flask. The sterility of the axenic larvae, as well as efficient colonization of the gnotobiotic larvae, was verified by PCR (see below). Five third-instar larvae were collected from each gnotobiotic treatment, and 10 axenic larvae were collected from three replicate flasks at the same time (5 days after hatching). Pooled larvae from each treatment were surface-sterilized by rinsing them once in sterile water, soaking in 70% ethanol for 10 min, and rinsing three times in sterile water. The larvae were then homogenized in Qiagen ATL extraction buffer, and DNA was extracted using the Qiagen DNeasy Blood and Tissue kit. The presence or absence of bacteria was qualitatively verified by PCR using the same primers listed above for bacteria identification. Homogenates

from the surface-sterilized larvae were plated to confirm that the added bacteria had colonized the larvae and that only a single morphological colony matching that of the input bacteria was present. The water in which gnotobiotic larvae developed was also plated to confirm that only the expected morphological colony was present. The axenic larval flasks were maintained for the duration of the experiment and manipulated in the same way to serve as negative controls. The amount of bacteria measured in the water of gnotobiotic treatments was not correlated to pupation rate (file S4).

### Selection of bacterial isolates for functional assays

Because cultivable bacteria only represent a small fraction of all bacteria present, and because specific bacterial isolates do not necessarily represent OTUs, the selection of isolates for functional assays was unrelated to the 16S metagenomics data. In particular, the choice of bacterial isolates did not depend on their relative abundance or habitat of origin. Instead, it was based on an arbitrary set of selection criteria described below. The original collection of 168 bacterial isolates was narrowed down to 37 isolates to test in an initial screen of pupation rate with the hypothesis that bacterial isolates that resulted in differences in larval growth kinetics would potentially induce phenotypic differences at the adult stage. The 37 test isolates were chosen on the basis of genetic dissimilarity to other isolates (<95% genetic similarity) and previously being associated with *Aedes* mosquitoes in the literature. To test the pupation rate of each of the 37 initial test isolates, individual colonies were inoculated into triplicate flasks of axenic larvae, as described above. The number of pupae was counted in each flask every day for 17 days. The list of 37 isolates was further narrowed down to 16 candidate isolates based on those that reached 60% pupation. Of the 16 candidate isolates, three isolates were chosen on the basis of differences in pupation rate (file S9) and differences in the cultivable bacterial composition found in adult midguts after 4 to 6 days in the insectary (file S10). On the basis of their full-length 16S sequence, two of the three isolates were assigned to *Salmonella* (*Ssp\_ivi*) and *Rhizobium* (*Rsp\_ivi*) genera. The third isolate (*Esp\_ivi*) was assigned to the Enterobacteriaceae family, but classification at the genus level was inconsistent among databases (alternatively *Salmonella*, *Escherichia*, or *Shigella*). Whereas *Escherichia* was previously found in wild *A. aegypti* specimens, and *Shigella* and *Rhizobium* were found in wild *A. albopictus* specimens, *Salmonella* was not previously reported to be associated with *Aedes* mosquitoes (20, 31). In all cases, colonies belonging to the bacterial genera that were added during the larval stage could not be recovered from the corresponding adult midguts. Even when the same bacterial genus was detected in adult midguts (file S10), the 16S sequence was distinct.

### Adult life-history traits

After adult emergence from the different gnotobiotic treatments, 18 to 20 females were placed into triplicate 1-pint cardboard cups and maintained under standard insectary conditions (27 ± 1°C; relative humidity, 75 ± 5%; 12:12-hour light/dark cycle) on a sugar diet. The number of dead mosquitoes was recorded daily for 60 days until >90% of mosquitoes had died. The wings of the individual females harvested in the second replicate of the vector competence experiment (see below) were kept for later analysis. Wing length was measured from the tip (excluding the fringe) to the distal end of the allula using an ocular micrometer and a dissecting microscope. When both wings were intact, the mean of the two wing lengths was used for the statistical analysis.

### Lysozyme-like activity of hemolymph

Antibacterial activity of the hemolymph was measured by a bacterial growth inhibition zone assay. In this assay, mosquito hemolymph was spotted onto an agar plate containing *M. luteus*, and the antibacterial activity of the hemolymph was measured by the area of visible bacterial clearance around the hemolymph sample. Five to 7 days after adult emergence from gnotobiotic treatments, hemolymph was collected from females and placed on agar plates seeded with *M. luteus*. To make the agar plate, 10 ml of agar solution [2× agar (BD BactoAgar, Becton, Dickinson and Company), freeze-dried *M. luteus* (5 mg/ml; Sigma-Aldrich), streptomycin (0.1 mg/ml; Sigma-Aldrich), and 67 mM potassium phosphate buffer (pH 6.4)] was plated, and 3-mm holes were punched in the solidified agar. Twenty females (two replicates of 10 females each) from each treatment were cold-anesthetized and stored on ice until hemolymph was collected. To collect hemolymph, 2 µl of anticoagulant solution [60% Schneider's medium (Sigma-Aldrich), 10% fetal bovine serum (FBS), and 30% citrate buffer (pH 4.5) (98 mM NaOH, 186 mM NaCl, 1.7 mM EDTA, and 41 mM citric acid)] was injected into the thorax using a finely drawn glass capillary and a bulb dispenser (Microcaps, Drummond Scientific Co.). Ten microliters of the anticoagulant solution was then injected into the abdomen, and hemolymph was collected through capillary action by placing a capillary tube next to the injection site. The hemolymph was immediately placed on ice and then deposited in the cutout holes on the agar plates. The plates were stored at 30°C for 24 hours, and the number of individuals with detectable *M. luteus* growth inhibition and the size of *M. luteus* growth inhibition zone were calculated. The size of *M. luteus* growth inhibition was determined by using ImageJ ([www.imagej.nih.gov/ij/](http://www.imagej.nih.gov/ij/)) to calculate the diameter of the clear zone. The diameter of the clear zone for the hemolymph samples was converted to lysozyme-like activity using a standard curve generated by spotting 10-fold serial dilutions of lysozyme (200 mg/ml; Sigma-Aldrich) and measuring the diameter of the clear zone using ImageJ.

### Vector competence

Following the gnotobiotic treatments, pupae were picked every day for 1 week, and adults were allowed to emerge under standard insectary conditions (27 ± 1°C; relative humidity, 75 ± 5%; 12:12-hour light/dark cycle). The adults were maintained in the insectary for 3 to 7 days after emergence on a standard sugar diet. Females were starved for 24 hours before the infectious blood meal. Vector competence assays were performed as previously described (68). Briefly, mosquitoes were experimentally exposed to a wild-type dengue virus serotype 1 isolate (KDH0026A) originally from Thailand (69). The isolate was passaged five times in *A. albopictus* C6/36 cells before its use in this study. The virus stock was diluted in cell culture medium (Leibovitz's L-15 medium + 10% heat-inactivated FBS + nonessential amino acids + 0.1% penicillin/streptomycin + 1% sodium bicarbonate) to reach a dose of  $2.4 \times 10^5$  FFU/ml in the first experiment and  $7.15 \times 10^5$  FFU/ml in the second experiment. One volume of virus suspension was mixed with two volumes of freshly drawn rabbit erythrocytes washed in distilled PBS and 60 µl of 0.5 M adenosine 5'-triphosphate. After gentle mixing, 2.5 ml of the infectious blood meal was placed in each of several Hemotek membrane feeders (Hemotek Ltd.) maintained at 37°C and covered with a piece of desalted porcine intestine as a membrane. After feeding, fully engorged females were sorted into 1-pint cardboard cups and maintained under controlled conditions (28 ± 1°C; relative humidity, 75 ± 5%; 12:12-hour light/dark cycle) in a climatic chamber for 14 days.

After 4 days (experiment 1) and 14 days (experiments 1 and 2), detection of dengue virus RNA was performed with a two-step reverse transcription PCR assay. Heads and bodies were separated from each other, and bodies were homogenized individually in 400  $\mu$ l of RAV1 RNA extraction buffer (Macherey-Nagel) during two rounds of 30 s at 5000 rpm (Precellys 24). Total RNA was extracted using the NucleoSpin 96 Virus Core Kit following the manufacturer's instructions (Macherey-Nagel). Total RNA was first reverse-transcribed to complementary DNA (cDNA) with random hexamers using M-MLV Reverse Transcriptase (Invitrogen). The cDNA was amplified by 45 cycles of PCR using the set of primers targeting the NS5 gene [5'-GGAAGGAGAAGGACTC-CACA-3' (forward) and 5'-ATCCTTGTATCCCATCCGGCT-3' (reverse)]. Amplicons were visualized by electrophoresis on 2.5% agarose gels.

In both experiments 1 and 2, the heads from infected bodies were titrated by standard focus-forming assay in C6/36 cells, as previously described (68). Briefly, heads were homogenized individually in 300  $\mu$ l of Leibovitz's L-15 medium supplemented with 2 $\times$  Antibiotic-Antimycotic (Life Technologies). C6/36 cells were seeded into 96-well plates, and each well was inoculated with 40  $\mu$ l of head homogenate and incubated for 1 hour at 28°C. Cells were overlaid with a 1:1 mix of carboxymethyl cellulose and Leibovitz's L-15 medium supplemented with 0.1% penicillin (10,000 U/ml)/streptomycin (10,000  $\mu$ g/ml), 1 $\times$  nonessential amino acids, 2 $\times$  Antibiotic-Antimycotic (Life Technologies), and 10% FBS. After 3 days of incubation, cells were fixed with 3.7% formaldehyde, washed three times in PBS, and incubated with 0.5% Triton X-100 in PBS. Cells were incubated with a mouse anti-dengue virus complex monoclonal antibody (MAB8705, Merck Millipore), washed three times with PBS, and incubated with an Alexa Fluor 488-conjugated goat anti-mouse antibody (Life Technologies). FFU were counted under a fluorescence microscope.

### Statistical analysis of mosquito phenotypes

All statistical analyses were performed in R v3.1.2 (www.r-project.org), unless where otherwise noted. Analysis of pupation rate was based on a three-parameter logistic model (Cumulative\_proportion =  $K/(1 + e^{-B(\text{time} - M)})$ ) describing the cumulative change in pupation rate over time for each condition using least-squares nonlinear regression with the minpack.lm R package (<https://cran.r-project.org/web/packages/minpack.lm/minpack.lm.pdf>). In this logistic model,  $K$  represents the saturation level of pupation rate (that is, the final pupation rate),  $B$  is the growth rate (that is, rate of change per unit time during the exponential phase), and  $M$  is the time at which the proportion of pupae equals 50% of the saturation level  $K$ . The extra sum-of-square  $F$  test was used to compare single parameters between two curves representing the cumulative pupation rate over time for two conditions. The  $P$  value was derived from the  $F$  test based on the  $F$  distribution and the number of degrees of freedom.

Survival data were analyzed using a time-to-event model and Kaplan-Meier estimator in the survival R package (<http://CRAN.R-project.org/package=survival>). Continuous variables (wing length, CFU counts, and FFU counts in the head) were analyzed using a full-factorial linear regression model and type III ANOVA, followed by verification of the normal distribution of the residuals. Binary traits (CFU prevalence, lysozyme-like activity prevalence, and vector competence binary phenotypes) were analyzed using a full-factorial logistic regression model and analysis of deviance.

### SUPPLEMENTARY MATERIALS

Supplementary material for this article is available at <http://advances.sciencemag.org/cgi/content/full/3/8/e1700585/DC1>

- fig. S1. Bacterial communities are richer (but not more diverse) in larval breeding site water than in mosquito midguts.
- fig. S2. Bacterial families differ between habitat and sample types.
- fig. S3. Dominant OTUs differ between habitat and sample types.
- fig. S4. No difference in the prevalence of midgut infection or systemic dissemination of dengue virus following different gnotobiotic treatments.
- file S1. Test statistics of richness and Shannon diversity index between sample and habitat type.
- file S2. Differentially abundant OTUs between sample types.
- file S3. Test statistics for larval growth rate and time to 50% pupation.
- file S4. Test statistics for the relationship between the amount of bacteria present in larval flasks and pupation rate.
- file S5. Habitat description of larval breeding sites sampled.
- file S6. Original oligonucleotide sequences used for 16S sequencing.
- file S7. Final oligonucleotide sequences used for 16S sequencing.
- file S8. Normalized OTU count table used for OTU-based analyses.
- file S9. Pairwise comparisons of growth rates (that is, slope of the exponential growth phase) among the 16 candidate bacterial isolates.
- file S10. Identity of cultivable bacteria present in midguts of adults exposed to different bacteria as larvae 4 to 6 days after emergence and maintained under standard insectary conditions as adults.

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**Acknowledgments:** We thank two anonymous reviewers for the helpful comments on an earlier version of the manuscript, N. Rahola and M. F. Ngangue for the assistance and expertise during the fieldwork, A. Fontaine for the help with the statistical analysis, C. Lallemand for the assistance with mosquito rearing, and all members of the Lambrechts laboratory for the insightful discussions. We also thank L. Ma for the technical assistance on the MiSeq and Centre d'Informatique pour la Biologie of Institut Pasteur for providing the computational resources. We are grateful to A. Ponlawat and T. Fansiri for the initial sampling of the mosquito population from Thailand. **Funding:** This study was supported by the French government's Investissement d'Avenir program, Laboratoire d'Excellence Integrative Biology of Emerging Infectious Diseases (grant ANR-10-LABX-62-IBEID), the French Agence Nationale de la

Recherche (MOSQUIBIOTA project, grant ANR-16-CE35-0004), the City of Paris Emergence(s) program in Biomedical Research, and the French Centre National de la Recherche Scientifique, Ecology of Health (ECOSAN) program (grant AFRICAÉDES). The Genomics Facility is a member of the "France Génomique" consortium (grant ANR-10-INBS-09-08). The funders had no role in the study design, data collection and analysis, decision to publish, or preparation of the manuscript. **Author contributions:** L.B.D., C.V.M., and L.L. conceived the study. L.B.D., D.J., D.A., and C.P. carried out the fieldwork. L.B.D., D.J., and I.M.-C. performed the experiments. C.B. supervised the high-throughput sequencing. L.B.D., G.M., S.V., A.G., and L.L. analyzed the data. L.B.D. and L.L. wrote the manuscript. All authors edited and commented on the manuscript. **Competing interests:** The authors declare that they have no competing interests. **Data and materials availability:** All data needed to evaluate the conclusions in the paper are present in the paper and/or the Supplementary Materials and/or have been deposited in the European Nucleotide Archive (accession number PRJEB16334). Materials are available upon request through a material transfer agreement. Additional data related to this paper may be requested from the authors.

Submitted 23 February 2017

Accepted 5 July 2017

Published 16 August 2017

10.1126/sciadv.1700585

**Citation:** L. B. Dickson, D. Jiolle, G. Minard, I. Moltini-Conclois, S. Volant, A. Ghazlane, C. Bouchier, D. Ayala, C. Paupy, C. V. Moro, L. Lambrechts, Carryover effects of larval exposure to different environmental bacteria drive adult trait variation in a mosquito vector. *Sci. Adv.* **3**, e1700585 (2017).