

Native microbiome impedes vertical transmission of *Wolbachia* in *Anopheles* mosquitoes

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Over evolutionary time, *Wolbachia* has been repeatedly transferred between host species contributing to the widespread distribution of the symbiont in arthropods. For novel infections to be maintained, *Wolbachia* must infect the female germ line after being acquired by horizontal transfer. Although mechanistic examples of horizontal transfer exist, there is a poor understanding of factors that lead to successful vertical maintenance of the acquired infection. Using *Anopheles* mosquitoes (which are naturally uninfected by *Wolbachia*) we demonstrate that the native mosquito microbiota is a major barrier to vertical transmission of a horizontally acquired *Wolbachia* infection. After injection into adult *Anopheles gambiae*, some strains of *Wolbachia* invade the germ line, but are poorly transmitted to the next generation. In *Anopheles stephensi*, *Wolbachia* infection elicited massive blood meal-induced mortality, preventing development of progeny. Manipulation of the mosquito microbiota by antibiotic treatment resulted in perfect maternal transmission at significantly elevated titers of the wAlbB *Wolbachia* strain in *A. gambiae*, and alleviated blood meal-induced mortality in *A. stephensi* enabling production of *Wolbachia*-infected offspring. Microbiome analysis using high-throughput sequencing identified that the bacterium *Asaia* was significantly reduced by antibiotic treatment in both mosquito species. Supplementation of an antibiotic-resistant mutant of *Asaia* to antibiotic-treated mosquitoes completely inhibited *Wolbachia* transmission and partly contributed to blood meal-induced mortality. These data suggest that the components of the native mosquito microbiota can impede *Wolbachia* transmission in *Anopheles*. Incompatibility between the microbiota and *Wolbachia* may in part explain why some hosts are uninfected by this endosymbiont in nature.

holobiome | dysbiosis | competitive exclusion |
microbe–microbe interactions | malaria

Bacteria in the genus *Wolbachia* are maternally transmitted *Rickettsia*-like endosymbionts that infect an estimated 40–69% of arthropod species (1, 2). In many cases, *Wolbachia* manipulate host reproduction to spread throughout arthropod populations (3). Incongruence between *Wolbachia* and host phylogenies indicate that horizontal transfer of the symbiont has been commonplace over evolutionary time (4, 5), enabling *Wolbachia* to invade new species. However, there is a poor understanding of barriers to horizontal transmission and why some species remain uninfected. An understanding of these factors is important from an evolutionary perspective given that *Wolbachia* influences speciation (6, 7), and from an applied perspective as *Wolbachia* is being transinfected into vector species for the control of arthropod-borne disease (8–10).

The ability to invade the host germ line is an important feature of *Wolbachia* biology that facilitates horizontal transmission, leading to the pervasive nature of this bacterium across invertebrate taxa. In order for *Wolbachia* to become established in a naïve host species, it must be acquired horizontally and successfully transmitted vertically (i.e., to offspring) to maintain the

infection in the population. Multiple mechanisms of *Wolbachia* horizontal transmission have been proposed, including cohabitation, hemolymph transfer, predation, and parasitoid infection (11–15). After microinjection into *Drosophila*, *Wolbachia* infects the stem cell niches in the germ line (16, 17), and both *Wolbachia*-derived and host factors appear to influence tropism and bacterial density during oogenesis (17–20). Alternatively, somatic tissue may act as a reservoir for *Wolbachia* infection of the developing oocyte (20–23). Although pathways of horizontal transmission have been characterized in some species, identification of barriers to vertical transmission of the acquired *Wolbachia* infection remains elusive.

Microbial conflict or incompatibility within arthropods is a potential barrier to transmission of heritable symbionts. Studies in the tick *Dermacentor variabilis* demonstrate competitive exclusion between maternally inherited bacteria. Transovarial transmission of *Rickettsia montanensis* (formerly *Rickettsia montana*) and *Rickettsia rhipicephali* is inhibited by infection with the reciprocal species (24). Similarly, infection exclusion has been observed in *D. variabilis* between conspecific strains of *Anaplasma marginale* where one strain inhibits the infection of the other (25). Competitive inter- and intraspecific microbial interactions have also been observed with *Wolbachia* (26, 27).

Significance

Factors influencing *Wolbachia* transfer into new species remain poorly understood. This is important as *Wolbachia* can influence speciation and is being developed as a novel arthropod-borne disease control approach. We show the native microbiota of *Anopheles* impede vertical transmission of *Wolbachia*. Antibiotic microbiome perturbation enables *Wolbachia* transmission in two *Anopheles* species. Mosquitoes with altered microbiomes do not exhibit blood meal-induced mortality associated with *Wolbachia* infection, suggesting that mosquitoes are killed by interactions between *Wolbachia* and other bacteria present in the mosquito. We identified *Asaia* as the bacterium responsible for inhibiting *Wolbachia* transmission, and partially responsible for blood meal-induced mortality. These results suggest that microbial interactions profoundly affect the host, and that microbiome incompatibility may influence distribution of *Wolbachia* in arthropods.

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Anopheles mosquitoes provide a unique system to examine microbial barriers to *Wolbachia* transmission. With few exceptions, Anophelines (which transmit the *Plasmodium* parasites that cause human malaria) are naturally uninfected with *Wolbachia* (28–31), suggesting the potential presence of innate barriers to infection in this genus. However, in vitro and in vivo studies indicate that *Wolbachia* are capable of infecting cultured *Anopheles* cells (32, 33), ex vivo cultured tissues (34), in vivo somatic tissue (35–37), and can stably infect the mosquito germ line (38). We investigated the ability of the native microbial community to influence vertical transmission of *Wolbachia* in *Anopheles* mosquitoes. We found that bacteria in the genus *Asaia* were responsible for inhibiting *Wolbachia* maternal transmission in this important mosquito genus.

Results

The wAlbB *Wolbachia* Strain Has a Greater Affinity for the *Anopheles* Germ Line Compared with wMelPop. For *Wolbachia* to stably infect a novel insect species, the bacteria must colonize the germ line so they can be maternally transferred to progeny to perpetuate the infection. We investigated transfer of two *Wolbachia* strains (wMelPop from *Drosophila melanogaster* and wAlbB from *Aedes albopictus*) into *Anopheles gambiae* and *Anopheles stephensi* using adult intrathoracic microinjection. Although many transfections have been accomplished using embryo microinjection (reviewed in ref. 9), this process may circumvent some barriers to vertical transmission (9). Injection of later life stages is more likely to resemble the process of horizontal transfer that occurs in nature (9, 11–15). After injection of the same number of *Wolbachia* bacteria into each mosquito, the wAlbB strain developed significantly higher germ-line titers compared with wMelPop, and invaded *A. gambiae* ovaries at significantly elevated titers compared with *A. stephensi* (Kruskal–Wallis, $P < 0.05$) (Fig. 1A). Similar results were observed for invasion of *A. gambiae* testes (Fig. S1). The carcass and ovarian density of wAlbB increased with time in *A. gambiae* (Kruskal–Wallis, $P < 0.004$), with ovarian density increase possibly due to replication within the ovary or more time to infect the germ line, or both (Fig. 1B).

FISH was performed on mosquitoes to visualize *Wolbachia* in the germ line and other tissues. The wAlbB strain was observed to infect numerous tissues including hemocytes, gut, pericerebral fat body, and brain (Fig. 1C–F), and was observed at low levels in the ovarian follicles of the mosquitoes, a location that is essential for maternal transmission (Fig. 1G). Subsequently, all further experiments were undertaken with the wAlbB strain.

Native *Anopheles* Microbiota Suppress Maternal *Wolbachia* Transmission.

To determine if microbial interactions influenced *Wolbachia* transmission in *Anopheles*, the microbiome of these mosquitoes was perturbed by antibiotic treatment. *Wolbachia* transmission was assessed in *A. gambiae* and *A. stephensi* mosquito lines reared continuously on a mixture of antibiotics (penicillin, streptomycin, gentamicin and kanamycin) and compared with a line reared on a conventional sugar diet. *A. gambiae* mosquitoes reared conventionally were capable of poorly transmitting the wAlbB strain of *Wolbachia* to their progeny, with only 10% of individuals being infected at very low titer. While fitness costs associated with *Wolbachia* infection in conventionally reared mosquitoes were evident in both species (affecting bloodfeeding and survival), they were extremely severe in *A. stephensi* and despite the high number of mosquitoes injected ($n = 2680$) no surviving adult offspring resulted from this line (Fig. 2). In stark contrast to conventionally reared mosquitoes, perfect vertical transmission (Fisher's exact, $P < 0.0001$) at significantly higher titers (Mann–Whitney U test, $P < 0.006$) was seen in antibiotic-treated *A. gambiae* (Fig. 2), and offspring survived from antibiotic-treated *A. stephensi*, of which ~90% were *Wolbachia*-infected (Fig. 2). Additionally, in *A. stephensi* both *Wolbachia* infection prevalence (Fisher's exact test, $P = 0.03$) and titer (Mann–Whitney U test, $P < 0.0001$) could be significantly boosted by repeated injections over multiple generations into the antibiotic-treated line (Fig. S2).

Antibiotic Treatment Reduces *Asaia* Infection in *Anopheles*. To determine the microbial composition of *Anopheles* lines reared on conventional sugar or antibiotics, we undertook high-throughput 16S amplicon sequencing for *A. gambiae* and *A. stephensi* (Fig. 3

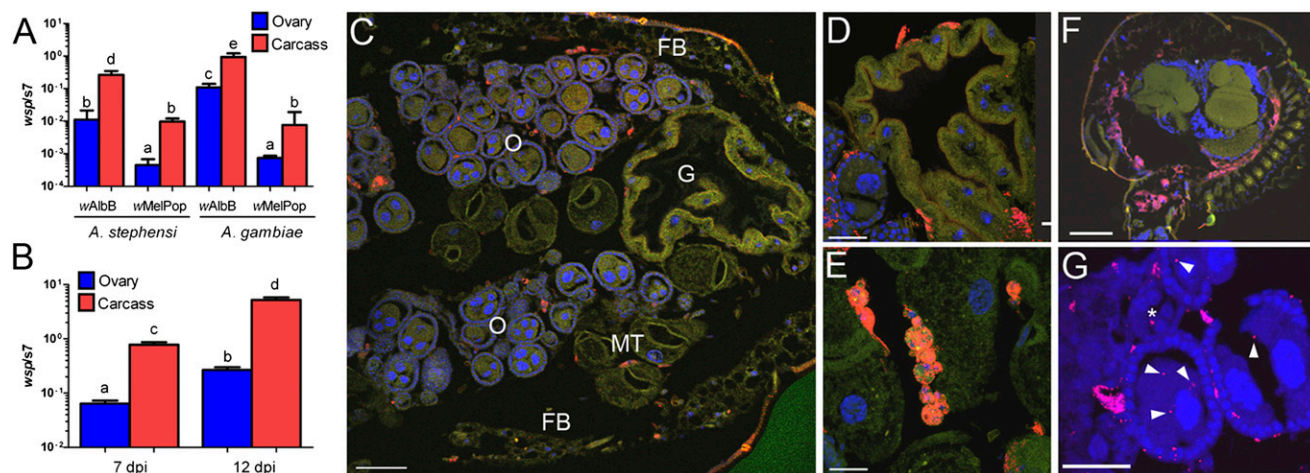


Fig. 1. Determining the optimal *Wolbachia* strain for vertical transmission in *Anopheles* mosquitoes. (A) *Wolbachia* density in microinjected mosquitoes assessed by qPCR at 7 dpi indicates the wAlbB strain infects *Anopheles* at significantly higher levels in both somatic and germ-line tissue. Approximately 9.3×10^6 bacterial cells were injected per mosquito. (B) The titer of wAlbB increases over time in *A. gambiae* somatic and germ-line tissue. Data for A and B were analyzed by Kruskal–Wallis test using the Dwass method for pairwise contrasts. The different letters (a–e) denote statistical significance [$P < 0.05$ in A and $P < 0.004$ in B]. Error bars in A and B represent SEM. (C) FISH performed on mosquitoes 17 dpi localizes the wAlbB infection in diverse tissues within the abdomen. O, ovaries; FB, fat body; G, gut; MT, malpighian tubule. Higher magnification of the (D) midgut, (E) hemocytes, (F) brain, and (G) ovarian follicles. The wAlbB strain infects the follicular epithelium of the oocyte and the nurse cells with the oocyte (white arrow heads in G). *Wolbachia* also infects the secondary follicles (asterisk in G). For ovarian follicles (G) mosquitoes were assessed 20 dpi. Red represents *Wolbachia*, blue the mosquito DNA, and green the mosquito tissue autofluorescence. (Scale bars: 300 μ m in C and 60 μ m in E–G.) FISH controls are available in Fig. S8.

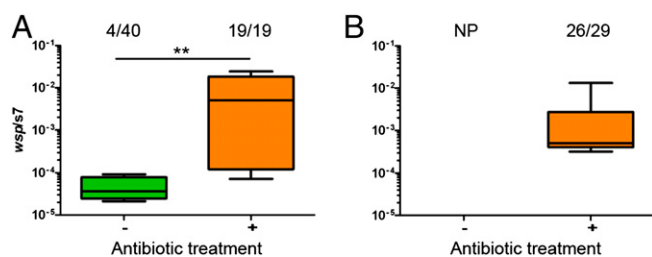


Fig. 2. Antibiotic perturbation of the microbiome enables vertical transmission of *Wolbachia* in *Anopheles* mosquitoes. (A) In *A. gambiae*, *Wolbachia* is transmitted poorly to mosquitoes reared on conventional sugar (no antibiotics) but the infection frequency (Fisher's exact test, $P < 0.0001$) and density (Mann–Whitney U test, $**P < 0.006$) increases significantly when mosquitoes are administered an antibiotic mixture. (B) In *A. stephensi*, no progeny (NP) were obtained from mosquitoes microinjected with *Wolbachia* reared on conventional sugar due to the severe fitness costs associated with infection. In mosquitoes administered an antibiotic mixture, *Wolbachia* was transmitted to 90% of offspring. Fractions represent the number of infected offspring over the total number. Green represents conventionally reared mosquitoes, whereas orange denotes antibiotic-treated mosquitoes. Box and whiskers represent data quartiles and range, respectively.

and Figs. S3–S6). While there were species-specific alterations in microbiome composition due to antibiotic treatment (Fig. 3, Fig. S3, and Table S1), only operational taxonomic units (OTUs) belonging to the bacterial family enterobacteriaceae and the bacterial genus *Asaia* showed significantly reduced levels for both mosquito species [nonparametric t test with Monte Carlo simulation comparing OTU frequencies between treatments: *A. gambiae* ($P < 0.01$) and *A. stephensi* ($P < 0.001$) (Table S1)]. *Asaia* showed one of the largest changes in absolute number of reads for all significantly regulated taxon categories (Table S1). Reductions in *Asaia* titers were independently verified by quantitative PCR (qPCR) analysis (Fig. 4A).

Microbiota–*Wolbachia* Interactions Kill Mosquitoes After a Blood Meal. *Asaia* bacteria naturally infect the *Anopheles* germ line and are vertically transmitted (39, 40), and thus may conflict with other maternally transmitted bacteria. This, together with the observed significant and large decrease in frequency of this taxon upon antibiotic treatment in both mosquito species, led us to investigate *Asaia* as a candidate bacteria involved in the interference of *Wolbachia* transmission. To assess *Wolbachia*–*Asaia* interactions in *Anopheles* mosquitoes, an antibiotic-resistant mutant of *Asaia* (herein *Asaia*^R) was created and supplemented to mosquitoes reared on antibiotics to create a gnotobiotic line. The *Asaia* infection status of all lines was confirmed with qPCR; *Asaia* was not detected in the unsupplemented antibiotic-treated line and no significant difference was found between *Asaia* infection frequency (Fisher's exact test, $P = 1.0$) or titer (ANOVA, $P = 0.06$) between conventionally reared and antibiotic-treated mosquitoes supplemented with *Asaia*^R (Fig. 4A). Given that we could not obtain offspring from the conventionally reared *A. stephensi* line, but could successfully raise progeny from the antibiotic-treated line post-*Wolbachia* injection (Fig. 2B), we assessed mortality trajectories of the three *A. stephensi* mosquito lines (conventionally reared, antibiotic treated, and antibiotic treated supplemented with *Asaia*^R) when infected with *Wolbachia*. Pre-blood meal, there was a modest but significant fitness cost of *Wolbachia* infection in conventionally reared *A. stephensi* compared with antibiotic-treated mosquitoes or antibiotic-treated mosquitoes supplemented with *Asaia*^R (ANOVA, $P < 0.0001$; Fig. 4B). Post-blood meal, there was a dramatic cost to *Wolbachia* infection in conventionally reared mosquitoes, with massive mortality in mosquitoes observed 2 d post-blood meal. Antibiotic treatment completely alleviated this mortality.

Antibiotic-treated mosquitoes supplemented with *Asaia*^R exhibited an intermediate mortality trajectory (ANOVA, $P < 0.0001$; Fig. 4C). These results indicate that interactions between *Wolbachia* and the native microbiota of *A. stephensi* induce blood meal mortality to which *Asaia* contributes in part, however the major microbial causative agent(s) of blood meal mortality have yet to be identified.

Microbiota Suppress *Wolbachia* Levels After a Blood Meal. Blood feeding drastically influences microbial abundance and composition in mosquitoes (41–43). Given that we observed severe blood meal-associated fitness effects in *Wolbachia*-infected *Anopheles*, *Wolbachia* and *Asaia* levels were assessed in *A. stephensi* mosquitoes pre- and post-blood feeding. A blood meal significantly reduced *Wolbachia* titers in the ovary and carcass of mosquitoes that possessed their natural microbiota compared with non-blood fed individuals [Mann–Whitney U test, $P < 0.001$ (ovaries) and $P < 0.01$ (carcass); Fig. 5A]. However, reduction in *Wolbachia* titers was not observed in antibiotic-treated lines [Mann–Whitney U test, $P = 0.18$ (ovaries) and $P = 0.93$ (carcass); Fig. 5B]. In contrast to *Wolbachia*, *Asaia* levels were significantly elevated in mosquitoes post-blood meal (Mann–Whitney U test, $P < 0.01$; Fig. 5B). No evidence was found for antibiotic treatment influencing *Wolbachia* levels in the female germ line (Mann–Whitney U test, $P = 0.1$; Fig. S7).

***Asaia* Inhibits *Wolbachia* Transmission in *Anopheles*.** To directly examine the effect of *Asaia* on *Wolbachia* transmission we compared transmission in antibiotic-reared *A. stephensi* lines with and without *Asaia* supplementation. In the antibiotic-treated line, *Wolbachia* was transmitted to the next generation with 83% of offspring infected. In contrast, *Wolbachia* transmission was completely abolished when *Asaia* was supplemented to female mosquitoes before injection (Mann–Whitney U test, $P < 0.0001$; Fig. 6).

Discussion

The microbiome affects the biology and physiology of the host across a broad range of eukaryotic taxa (44–46). In mammals, microbial interactions (particularly competitive exclusion) shape the composition of the microbiome (47). Similarly, the insect

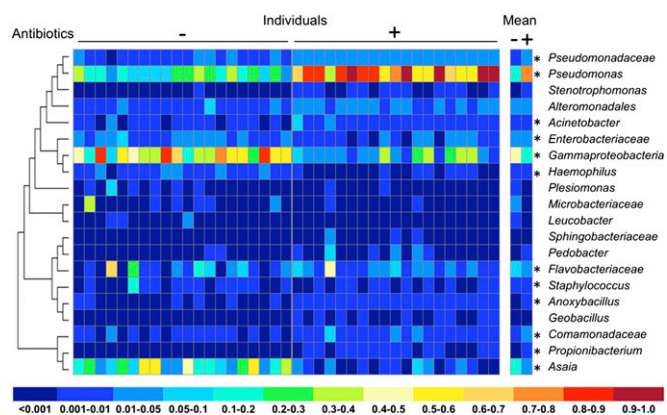


Fig. 3. Microbiome analysis of *A. stephensi* mosquitoes reared on conventional sugar (–) compared with those on an antibiotic mixture (+). OTUs were grouped by genus (where possible) or higher rank, and the relative abundance in individual samples calculated. The mean relative abundance per treatment is also shown. Asterisks denote presence of OTUs within that taxon grouping that significantly change in frequency (read count) between treatments (nonparametric t test with Monte Carlo simulation; see Table S1 for OTU-specific P values). A maximum likelihood phylogenetic tree was constructed based on the alignment of representative 75-nt OTU sequences for each taxonomic group. See Fig. S3 for a comparison of the relative abundance of bacterial taxa in *A. gambiae*.

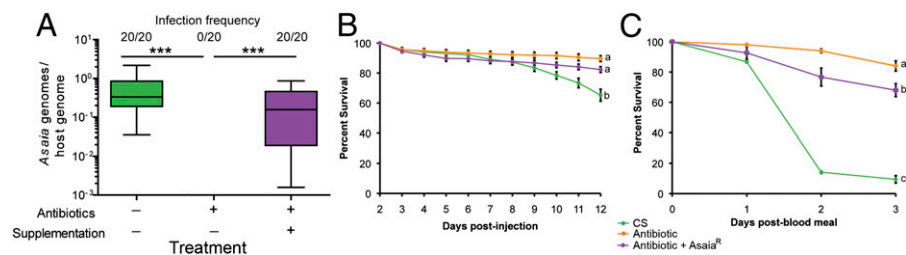


Fig. 4. *Asaia* density and mosquito longevity in conventionally reared, antibiotic-treated, and *Asaia*-supplemented antibiotic-treated *A. stephensi*. (A) Levels of *Asaia* in control mosquitoes, antibiotic-treated mosquitoes, and antibiotic-treated mosquitoes supplemented with *Asaia*^R. Treating mosquitoes with an antibiotic mixture eliminates *Asaia*, whereas supplementation with *Asaia*^R in a sugar meal reestablishes the infection (ANOVA, *** $P < 0.0001$). *Asaia* levels in conventionally reared and *Asaia*^R supplemented mosquitoes are not significantly different (ANOVA, $P = 0.06$). Fractions represent the number of infected mosquitoes over the total number. Box and whiskers represent data quartiles and range, respectively. (B and C) Mosquito mortality trajectories after *Wolbachia* injection in mosquito lines pre- (B) and post-blood meal (C). Conventionally reared mosquitoes suffer slight fitness costs after injection and severe mortality after a blood meal, whereas antibiotic-treated mosquitoes do not suffer elevated mortality. Antibiotic-treated mosquitoes supplemented with *Asaia*^R exhibit a modest increase in mortality post-blood meal (ANOVA; letters a–c denote statistical significance $P < 0.05$). Green represents conventionally reared mosquitoes, orange the antibiotic-treated mosquitos, and purple the antibiotic-reared mosquitos supplemented with *Asaia*^R. Error bars in B and C represent SEM.

microbiome is important for host homeostasis, development, and immunity (48–50) and has recently been implicated to influence speciation (51). Although the microbiota of vector arthropods is known to be a potent modulator of pathogen vector competence (52, 53), little is known regarding bacterial interactions within these medically important insects. Our data suggest the native microbiota of *Anopheles* mosquitoes can impede transmission of the maternally inherited symbiont *Wolbachia*. Mosquitoes with an antibiotic-perturbed microbiome transmitted *Wolbachia* to their offspring and had reduced fitness costs associated with *Wolbachia* infection. In mosquitoes that possessed their native microbiota, decreases in *Wolbachia* transmission may be due to microbiome-induced reductions in *Wolbachia* titers post-blood meal, similar to what has been observed in naturally *w*Flu-infected *Aedes fluviatilis* mosquitoes (54).

Competitive exclusion between bacterial species can shape the composition of arthropod microbiomes (24–27). Using a combination of high-throughput sequencing and bacterial supplementation, we identified the bacterium *Asaia* as a specific agent inhibiting *Wolbachia* transmission in *Anopheles*. Recently, *Wolbachia* sequences were detected at low frequency in field populations of *A. gambiae* (55). High-throughput sequencing of the 16S rRNA gene amplified from the DNA extracted from the germ lines of 30 mating couples found one male that contained *Wolbachia* reads (55). Although lack of statistical power precludes definitive conclusions, examination of these data identified no *Asaia* sequence reads in the *Wolbachia*-infected individual, consistent with the hypothesis that *Asaia* acts competitively with *Wolbachia* infection in *Anopheles*. However, *Asaia* was also absent from other mosquitoes that were not infected with *Wolbachia* (55). A larger sample size is required to examine any correlation between *Wolbachia* and *Asaia* in potential natural *A. gambiae* infections. Our results provide a hypothesis to explain why the majority of *Anopheles* mosquitoes lack *Wolbachia* in nature, and more broadly, may in part explain the distribution of *Wolbachia* infections across taxa.

Strategies which exploit *Asaia* and *Wolbachia* are currently being devised for malaria control (38, 39). Further work is needed to determine if these approaches are complementary or incompatible, and whether the presence of *Asaia* in field populations will hinder the spread of *Wolbachia* into natural populations. Although a stable *Wolbachia* infection has been created in the Asian malaria mosquito *A. stephensi* (38), other important *Anopheles* vectors are yet to be transfected with *Wolbachia*. Eliminating *Asaia* may facilitate the development of *Wolbachia* infections in these other vectors. The creation of *Anopheles* mosquito lines transfected with a variety of *Wolbachia* strains will be worthwhile, given that some *Anopheles*–*Wolbachia* strain combinations may not be suitable for

malaria control due to their propensity to increase certain *Plasmodium* parasites (36, 56, 57).

Materials and Methods

***Wolbachia* Culture and Extraction.** The *w*AlbB and *w*MeIPop strains of *Wolbachia* were purified from infected *A. gambiae* Sua5B and Mos55 cells, respectively, according to previously published procedures (37). Purified *Wolbachia* were stained using the Live/Dead BacLight Bacterial Viability Kit (Invitrogen). *Wolbachia* were quantified using a hemocytometer under a fluorescence microscope (Olympus) to determine the density of viable cells.

Mosquito Rearing and Antibiotic Treatment. *A. gambiae* (Keele strain) and *A. stephensi* (Liston strain) mosquitoes were reared on a conventional 10% (wt/vol) sugar diet and blood fed with an artificial feeder on expired human blood for reproduction. Antibiotic-treated lines of each mosquito species were reared with 10% sugar containing an antibiotic mixture of penicillin–streptomycin (10 units/mL, 10 μ g/mL), gentamicin (15 μ g/mL), and kanamycin (200 μ g/mL) (58). Mosquito lines were continuously reared on the antibiotic mixture and all experiments in these lines were conducted after a minimum of three generations of antibiotic treatment. *Wolbachia* replication dynamics are not affected by kanamycin, penicillin or streptomycin, however *Wolbachia* is moderately susceptible to gentamicin (59). Therefore,

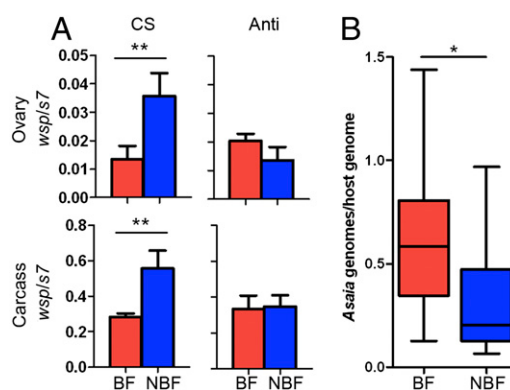


Fig. 5. Bacterial densities are modulated by blood feeding in *A. stephensi*. (A) By qPCR, *Wolbachia* levels in the ovary and the carcass significantly decrease after a blood meal in mosquitoes reared on conventional sugar (CS) [Mann–Whitney U test, *** $P = 0.0015$ (ovary) and ** $P = 0.0178$ (carcass)]. This reduction is abolished when mosquitoes are reared on an antibiotic mixture (Anti) [Mann–Whitney U test, $P = 0.18$ (ovary) and $P = 0.93$ (carcass)]. Error bars represent SEM. (B) In conventionally reared mosquitoes, blood feeding significantly increases *Asaia* levels compared with non-blood fed mosquitoes (Mann–Whitney U test, * $P < 0.01$). BF, blood fed (red); NBF, non-blood fed (blue). Box and whiskers represent data quartiles and range, respectively.

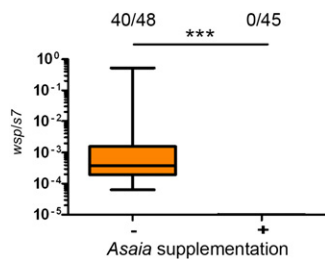


Fig. 6. *Asaia* inhibits *Wolbachia* transmission in *A. stephensi* mosquitoes. Supplementing *Asaia*^R to antibiotic-treated *A. stephensi* abolishes *Wolbachia* transmission to offspring. Transmission is significantly different between nonsupplemented (orange) and supplemented mosquito lines that were reared on antibiotics (Fisher's exact test, *** $P < 0.0001$). Fractions represent the number of infected offspring over the total number. Box and whiskers represent data quartiles and range, respectively.

gentamicin was removed from the antibiotic mixture before *Wolbachia* infection. Two to 5 d postemergence, mosquitoes were anesthetized on ice and injected with *Wolbachia* ($\sim 10^7$ bacteria per mosquito) according to a previously established methodology (37). Postinjection, mosquitoes were maintained at 28 °C with access to 10% sucrose (with or without antibiotics).

qPCR. Ovaries were dissected from anesthetized mosquitoes under a dissecting microscope (Olympus) and DNA extracted either using QIAamp DNA Micro Kits (Qiagen) or DNeasy blood and tissue extraction kits (Qiagen) following the manufacturer's protocols. qPCR was performed in triplicate on a Rotor Gene Q (Qiagen) using the Rotor Gene SYBR Green PCR Kit (Qiagen) according to Hughes et al. (35) and *Wolbachia* to host genome ratios were calculated using Qgene (60).

FISH. Mosquitoes were fixed in acetone then embedded in paraffin wax and sectioned with a microtome. Slides were dewaxed with three successive xylene washes for 5 min, followed by 5-min washes with 100% ethanol then 95% ethanol before treatment with alcoholic bleach (6% H₂O₂ in 80% ethanol) for 3 d to minimize autofluorescence. FISH was completed as previously described (35). Images were captured with an LSM 510 META confocal microscope (Zeiss) and Olympus FV1000 Laser Scanning Confocal Microscope (Olympus). Images were processed using LSM image browsers (Zeiss), FV10-ASW Version 3.0 (Olympus), and Photoshop 7.0 (Adobe) software. Uninfected and no-probe controls were performed and are available in Fig. S8.

Microbiome Analysis. The microbiomes of conventionally reared and antibiotic-treated *Anopheles* were characterized and compared using barcoded high-throughput amplicon sequencing of the bacterial 16S ribosomal gene. Whole conventionally reared *A. gambiae* ($n = 5$) and *A. stephensi* ($n = 20$), and antibiotic-treated *A. gambiae* ($n = 5$) and *A. stephensi* ($n = 19$) were surface sterilized by a wash in 20% bleach followed by three washes in 100% ethanol to reduce external contaminant bacteria. Total genomic DNA from whole mosquitoes was extracted following the Marriott extraction protocol (61), followed by washes and column elution for improved purity ratios (Powersoil DNA Elution kit; MO BIO). Initial preliminary sequencing was conducted on *A. gambiae*, where DNA samples underwent barcoded PCR of the 16S V3–V4 region followed by sequencing on the 454 Junior platform (Roche Inc). More in-depth sequencing to allow finer-scale changes to be detected was conducted on *A. stephensi* where DNA samples underwent barcoded PCR of the 16S V6 region followed by sequencing on the MiSeq platform (Illumina Inc.). For both species, primers and adaptors were subsequently removed from reads, the reads separated by sample barcode, and then the quality (minimum Q20) and length of the reads filtered. Sequences were clustered into OTUs at the 97% similarity level by reference-based picking with Quantitative Insights into Microbial Ecology (QIIME) 1.8.0 (62) implementation of UCLUST (63), against the 13_8 release of the Greengenes

16S database (64), with the remaining sequences clustered de novo. The QIIME default classifier UCLUST was used to assign taxonomy to OTUs. Only OTUs that contained more than five sequences were retained for subsequent analyses, reducing the likelihood of inclusion of OTUs formed through sequencing error. For beta diversity (between sample/treatment) measures, samples were standardized by randomly selecting a set number of sequences per sample. This value was chosen to equate that present in the sample with the lowest number of sequences (1,434 sequences for *A. gambiae* and 16,165 for *A. stephensi*), thus allowing inclusion of all samples but maximizing sequencing depth. For significance tests (nonparametric t test with Monte Carlo simulation) of OTU frequencies between treatments, OTUs were further filtered to remove low-frequency taxa. The filter threshold was taken as 1% of the mean sample sequence depth/read count (thresholds: 90 sequences for *A. gambiae* and 1,370 for *A. stephensi*). Microbial composition, rarefaction, diversity, and OTU frequency changes between treatments were analyzed using QIIME 1.8.0 pipelines. *Asaia* densities in all mosquito lines were corroborated by qPCR ($n = 20$) (39).

***Wolbachia* Transmission Experiments.** At 17–20 d post-*Wolbachia* injection, mosquitoes were provided with access to a blood meal and then given access to an oviposition site. For each experiment, both the treatment and control were injected with the same density of *Wolbachia* and blood fed at the same time postinjection. After mosquitoes oviposited, eggs were washed once with 10% bleach and three times with water, hatched in 1.5-L trays, and fed dried fish food. Emerging mosquitoes were collected and assayed by qPCR for *Wolbachia*. Transmission assays on antibiotic-treated mosquitoes were replicated at least twice. A kanamycin-resistant *Asaia* mutant was created by culturing an *A. stephensi* *Asaia* isolate on kanamycin (100 µg/mL) Gly media (25 g/L glycerol, 10 g/L yeast extract). The resistant *Asaia* was cultured in Gly media (kanamycin 100 µg/mL) for 2 d at 30 °C, supplemented to mosquito larvae (1×10^6), and provided to adults in the sugar. Mosquitoes were reared on 10% sugar containing an antibiotic mixture of penicillin–streptomycin (10 units/mL, 10 µg/mL) and kanamycin (200 µg/mL).

Mortality Assessment. The mortality dynamics of mosquitoes pre- and post-blood meal were conducted as previously described (35, 36). *wAlbB* was injected into mosquitoes reared on conventional sugar, reared continuously on antibiotics, or reared continuously on antibiotics but supplemented with *Asaia*^R as described above. After *Wolbachia* injection, mosquitoes were placed into cups (60 per cup) with 6–10 replicate cups per treatment. Mortality of mosquitoes was monitored every 24 h for 12 d. After 12 d, mosquitoes were given a blood meal through a membrane feeder, and blood fed mosquitoes were sorted into new cups (30 mosquitoes per cup, 5 replicate cups per treatment), and mortality assayed for 3 d. Data were analyzed by ANOVA with Tukey's correction for multiple comparisons.

Ovary Titer Analysis Pre- and Post-Blood Meal. *Wolbachia*-injected *A. stephensi* mosquitoes were either given a blood meal as described above at 5 d post-injection (dpi) or continuously reared on 10% sucrose. At 8 dpi ovaries were dissected from both blood-fed and sugar-fed mosquitoes ($n = 10$). DNA was extracted from ovaries and carcasses and used as the template for qPCR as described above. To determine the effect of the microbiota on *Wolbachia* ovary titers after a blood meal, experiments were repeated with antibiotics supplemented in the 10% sucrose solution (penicillin–streptomycin and kanamycin at previously described concentrations). Data were analyzed by Mann-Whitney *U* test. All experiments were replicated twice.

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