Supporting Information

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SI Materials and Methods

Mosquito Collection. Mosquitoes were collected in the villages of Dangassa (12.14°N, 8.20°W) and Kenieroba (12.11°N, 8.33°W), southwest of Bamako, Mali, along the Niger River in May–January 2010–2011, October–November 2015, and July–August 2016 (Fig. 1). Houses were sprayed with pyrethrum, and female mosquitoes were captured and desiccated using Eppendorf tubes with silica gel.

Homogenization of Samples for *Plasmodium* Detection by Anti-CSP ELISA and Genomic DNA Extraction. Head-thorax segments were dissected and homogenates were prepared as described (42). Briefly, samples were homogenized with a plastic pestle in 50 μL of blocking buffer (5% casein, 50 mM EDTA, PBS pH 7.4) (BB-EDTA), supplemented with 5% Nonidet P-40 (BB-Nonidet P-40). The pestle was rinsed by adding 200 μL of BB-EDTA, bringing the final homogenate volume to 250 μL. *Plasmodium* infection was detected using 50 μL of the homogenate to detect the *P. falciparum* CSP using an anti-sporozoite monoclonal antibody with an ELISA kit (BEI Resources) with samples done in duplicate (100 μL of total homogenate). The rest of the homogenate (150 μL) was immediately frozen in dry ice and stored at −80 °C.

Genomic DNA Extraction and Quantitation of *P. falciparum* and *Wolbachia* Infection in Field-Collected Mosquitoes. Genomic DNA was extracted from 150 μL of homogenate. The homogenates were incubated with 3 mg/mL proteinase K at 56 °C for 1 h, and genomic DNA (gDNA) was purified using phenol/chloroform extraction, followed by two chloroform extractions. The aqueous layer was transferred to a new tube, 0.1 volumes of 0.3 M sodium acetate and 0.7 volumes of isopropanol were added, and DNA was precipitated by centrifugation (16,000 × *g* for 15 min). The pellet was gently washed twice with 100 μL of 70% ethanol (16,000 × *g* for 5 min) and resuspended in 200 μL of Tris-EDTA buffer. To further purify the DNA, samples were reprecipitated (with 0.1 volumes of 0.3 M sodium acetate and 0.7 volumes of isopropanol) and gently washed twice with 70% ethanol. DNA pellets were air-dried for 30–60 min and resuspended in 30 μL of TE. *Wolbachia* was detected by PCR (forward: 5′-CATACCTATTCGAAGGGATAG-3′, reverse: 5′-AGCTTCGAGTGAAACCAATTC-3′) (25) using the following amplification conditions: 10 min at 95 °C, followed by 45 cycles of 45 s at 95 °C, 45 s at 60 °C and 1 min at 72 °C, followed by 10 min at 72 °C. Nested PCR was used to detect *Wolbachia* using *Wolbachia*-specific primers targeting 16S rRNA (forward: 5′-GAAGGGATAGGGTCGGTTCG-3′, reverse: 5′-CAATTCC-CATGGCGTGACG- 3′) under the following conditions: 15 min at 95 °C, followed by 35 cycles of 15 s at 95 °C, 25 s at 66 °C, and 1 min at 72 °C, followed by 5 min at 72 °C (23). PCRs were performed using Taq DNA Polymerase, native kits (ThermoFisher) with 500 nM forward and reverse primers. The specificity of the PCRs was confirmed by Sanger sequencing.

***Wolbachia* Phylogenetic Analysis and MLST Typing.** For phylogenetic analysis, Rickettsiales-specific primers targeting a variable region of the 16S rRNA were used [forward: 5′-CAGACGGGTGAG-TAATG(C/T)ATAG-3′, reverse: 5′-TATCACTGGCAGTTTC-CTTAAAG-3′] under the following conditions: 10 min at 95 °C, followed by 45 cycles of 45 s at 95 °C, 45 s at 64 °C and 1 min at 72 °C, followed by 5 min at 72 °C (25). PCRs were performed using Taq DNA Polymerase, native kits (ThermoFisher) with 500 nM forward and reverse primers. Following Sanger sequencing, downstream analysis was performed using the Geneious R10 software platform (Biomatters). Sequenced PCR products were blasted against the National Center for Biotechnology Information (NCBI) nucleotide database. The top 500 results were downloaded and manually inspected to remove redundancy. Reference *Rickettsia* sequences were added and sequence alignment was performed using the MUSCLE algorithm. A phylogenetic tree was reconstructed using a Tamura-Nei genetic distance model. For MLST typing, primer sequences and PCR conditions were used as instructed by the *Wolbachia* MLST Database (26). PCRs were performed using Taq DNA Polymerase, native kits (ThermoFisher) with 500 nM forward and reverse primers.

***Wolbachia* and *Plasmodium* Detection and by qPCR.** *Wolbachia* levels within mosquito genomic DNA samples were analyzed by SYBR green qRT-PCR. *Wolbachia*- and *Plasmodium*-specific primers against 16S (forward: 5′-CATACCTATTCGAAGGGATAG-3′, reverse: 5′-TTGCGGGACTTAACCCAACA-3′) and 28S (forward: 5′-GTGGCCTATCGATCCTTTA- 3′, reverse: 5′-GCG-TCCCAATGATAGGAAGA-3′) rRNA were used, respectively. The amount of gDNA template was normalized based on the levels of *A. gambiae* S7 rRNA gene in each sample (forward: 5′-GCGTCCCAATGATAGGAAGA-3′, reverse: 5′-GCTGCAA-ACTTCGGCTATTC-3′). The PCRs were performed under the following conditions: 15 min at 95 °C, followed by 45 cycles of 15 s at 94 °C, 20 s at 60 °C and 30 s at 72 °C, followed by 5 min at 72 °C. PCRs were performed using DyNAmo HS SYBR Green qPCR Kit (ThermoFisher) with 300 nM forward and reverse primers using a CFX96 system (Bio-Rad). gDNA isolated from females of the NIH *A. gambiae* G3 colony, that is not infected with *Wolbachia*, was used as positive and negative control for the qPCR assays. Amplification of the *A. gambiae* S7 gene was confirmed, as well as the lack of *Wolbachia* amplification. Cycles threshold (Ct) values beyond 40 cycles were considered as negative, and the limit of detection was set at a point where the correlation between technical replicates was very high (R^2 = 0.9978, $P < 0.0001$).

Establishment of *Wolbachia*-Infected *A. coluzzii* Colony. Females were collected from Dangassa and allowed to lay eggs in individual containers. Eggs were allowed to hatch and mothers were genotyped as either *A. gambiae* (S molecular form) or *A. coluzzii* (M molecular form), as previously described (43). *A. coluzzii* larvae from 23 females were pooled and reared. Upon emergence, adult mosquitoes were mixed for three generations with *A. coluzzii* males from a colony kept at the Laboratory of Malaria and Vector Research, NIH. Mosquitoes were treated with Penicillin-Streptomycin (Sigma-Aldrich), because some bacteria from the microbiota, such as *Asaia*, have been reported to reduce vertical transmission of *Wolbachia* anophelines (44). Briefly, mosquitoes received 100 units/mL Penicillin and 100 μg/mL Streptomycin in water for 2 d before and 2 d after blood feeding. Sucrose was provided as a dry sugar cube, and mosquitoes were allowed to lay eggs in a wet filter paper.

Experimental Infection of Mosquitoes with *P. falciparum*. Mosquito females were artificially infected by membrane feeding with *P. falciparum* NF54 gametocyte cultures. Gametocytogenesis was induced as previously described (45) and 14- to 16-d mature gametocyte cultures (stages IV and V) were used to feed mosquitoes using membrane feeders at 37 °C for 30 min. *Wolbachia* and *Plasmodium* infection levels were analyzed by qPCR using dissected midgut or head-thorax gDNA samples 8–10 or 18–21 d after feeding, respectively. Midgut gDNA was extracted using

the DNEasy Blood and Tissue Kit (Qiagen) following lysozyme and proteinase K treatment. Head-thorax gDNA was extracted from dissected segments. Samples were homogenized in 50 μL of TE and incubated at 65 °C for 30 min. Samples were incubated with 20 mg/mL lysozyme at 37 °C for 1.5 h, and 1.3 mg/mL proteinase K at 56 °C for 1.5 h. Following the addition of 0.85 M potassium acetate, samples were incubated on ice for 30 min and centrifuged 16,000 × *g* for 15 min. The supernatant was transferred, incubated at room temperature for 5 min with 0.7 volumes of isopropanol and centrifuged 16,000 × *g* for 15 min. The pellet was washed twice with 100 μL of 70% ethanol, air dried for 30–60 min, and resuspended in 20 μL of TE.

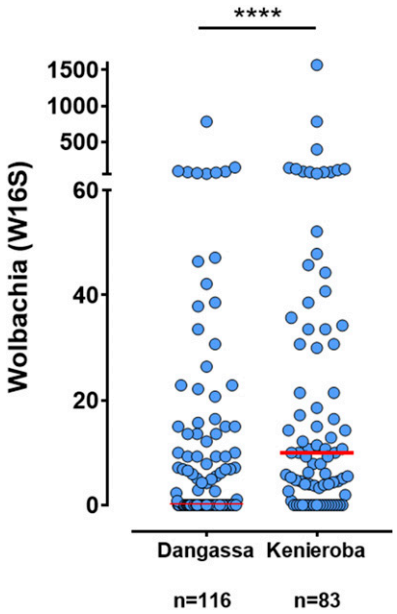

Fig. S1. *Wolbachia* levels in field-collected mosquitoes. *A. gambiae s.l.* females were collected in the villages of Dangassa (12.14°N, 8.20°W) and Kenieroba (12.11°N, 8.33°W), southwest of Bamako, Mali, in October–November 2015, and *Wolbachia* levels were determined by qRT-PCR. The amount of gDNA template was normalized based on the levels of *A. gambiae* S7 rRNA gene in each sample. Sample value distributions were compared using the Mann–Whitney test and are indicated by the red line (n = number of samples, ****$P < 0.0001$).

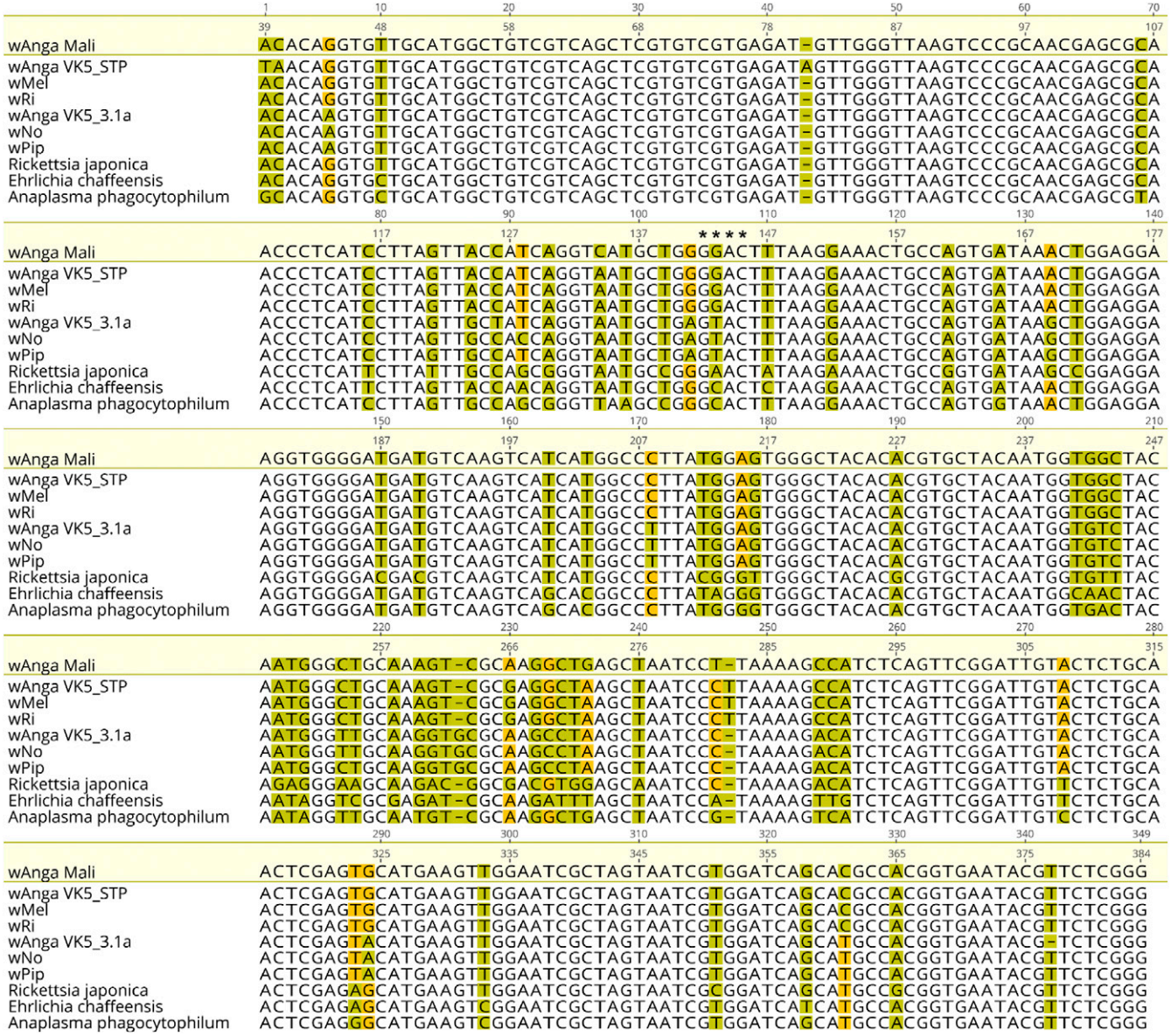

Fig. S2. Alignment of a conserved region of the 16S rRNA gene using *Wolbachia*-specific primers (W16S-Spec). Asterisks denotes the nucleotide residues that encode an RsaI restriction site, a signature feature of *Wolbachia* supergroup B.

Fig. S3. Sequence alignment of the deduced amino acid sequence of the region of the *hcpA* gene from *Wolbachia* and other non-*Wolbachia* pre-teobacterium. These sequences are used in the MLST scheme, a universal genotyping tool for *Wolbachia*.

Fig. S4. Sequence alignment of the deduced amino acid sequence of the region of the *fbpA* gene from *Wolbachia* and other non-*Wolbachia* proteobacterium. These sequences are used in the MLST scheme, a universal genotyping tool for *Wolbachia*.

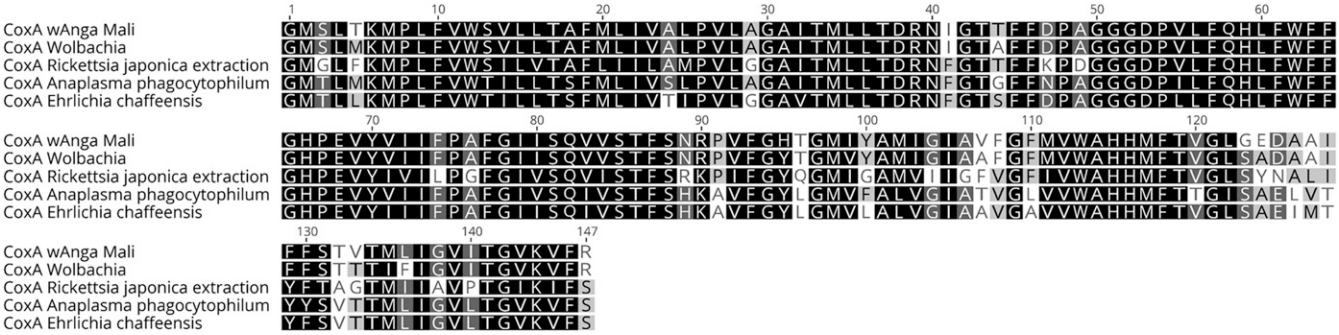

Fig. S5. Sequence alignment of the deduced amino acid sequence of the region of the *coxA* gene from *Wolbachia* and other non-*Wolbachia* proteobacterium. These sequences are used in the MLST scheme, a universal genotyping tool for *Wolbachia*.

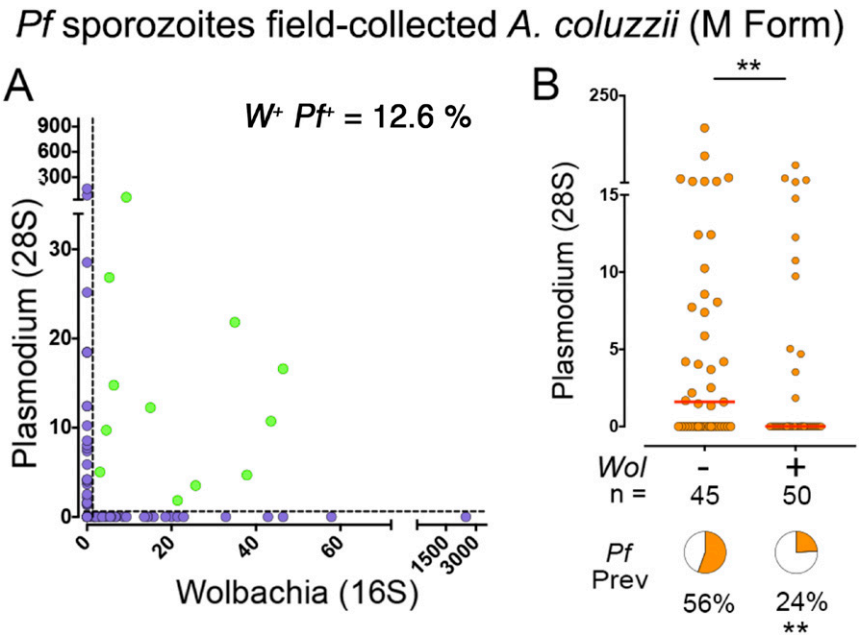

Fig. S6. Effect of naturally occurring *Wolbachia* infection on *Plasmodium* sporozoite infection in field-collected *A. coluzzii*. *Wolbachia* and *Plasmodium* levels in field-collected *A. coluzzii* were determined by qRT-PCR. (*A*) Correlation between *Wolbachia* and *Plasmodium* levels. The relative abundance of coinfected mosquitoes (W^+Pf^+) is indicated. (*B*) Levels of *Plasmodium* sporozoite infection in *Wolbachia*-infected and uninfected females. Each data point represents the level of *Plasmodium* infection in a single mosquito, and medians are indicated by the line. Sample value distributions were compared using the Mann–Whitney test. Pie charts represent the prevalence of *Plasmodium* sporozoite infection in *Wolbachia*-infected and uninfected samples. The prevalence was compared using the χ^2 test (n = number of samples). $^{**}P < 0.01$.

Table S1. Number of mosquitoes collected in 2010 from six Malian villages and the number of samples positive for anti-CSP ELISAs from head-thorax mosquito homogenates

Location	*Plasmodium* +	Total	*Plasmodium* prevalence
Kenieroba	46	3,788	1.21
Fourda	5	851	0.58
Dangassa	89	3,280	2.71
Dangassa-somonosso	6	475	1.26
Niaganabougou	51	4,812	1.06
Niaganabougou-somonosso	8	1,815	0.44

Table S2. Primer sequences used for PCR amplifications

Primer	Forward primer	Reverse primer
W16S-Spec	CATACCTATTCGAAGGGATAG	AGCTTCGAGTGAAACCAATTC
W16S-Nested	GAAGGGATAGGGTCGGTTCG	CAATTCCCATGGCGTGACG
W16S-WE	CAGACGGGTGAGTAATGYATAG	TATCACTGGCAGTTTCCTTAAAG
W16S-qPCR	CATACCTATTCGAAGGGATAG	TTGCGGGACTTAACCCAACA
gatB	GAKTTAAAYCGYGCAGGBGTT	TGGYAAYTCRGGYAAAGATGA
coxA	TTGGRGCRATYAACTTTATAG	CTAAAGACTTTKACRCCAGT
hcpA	GAAATARCAGTTGCTGCAAA	GAAAGTYRAGCAAGYTCTG
ftsZ	ATYATGGARCATATAAARGATAG	TCRAGYAATGGATTRGATAT
fbpA	GCTGCTCCRCTTGGYWTGAT	CCRCCAGARAAAAYYACTATTC
S7	AGAACCAGCAGACCACCATC	GCTGCAAACTTCGGCTATTC
P28S	GTGGCCTATCGATCCTTTA	GCGTCCCAATGATAGGAAGA

Table S3. *w*Anga-Mali sequence homology to specific regions of the *coxA*, *hcpA*, and *fbpA* genes

Gene	*Wolbachias*, %	*A. phagocytophilum*, %	*R. japonica*, %	*E. chaffeensis*, %
hcpA	91	55	46	56
fbpA	93	59	—	65
coxA	93	73	70	74

These sequences are used to classify Wolbachia strains using the multilocus sequence typing (MLST) scheme, a universal genotyping tool for *Wolbachia* (*coxA*, cytochrome *c* oxidase, subunit I; *fbpA*, fructose-bisphosphate aldolase; *hcpA*, conserved hypothetical protein;). Different *Wolbachia* strains that infect other hosts have the highest nucleotide sequenced homology to these genes in *w*Anga-Mali [*Wolbachia* from *Megalothorax incertus* for *hcpA* (91%); *Brugia pahangi* for *fbpA* (93%); and *Thalassaphorura houtanensis* for *coxA* (93%)]. The sequence homology to other Rickettsiales is lower and is indicated in the table. Sequence comparisons were done at the amino acid levels (blastx) using BLAST/NCBI/NIH.