Effect of naturally occurring *Wolbachia* in *Anopheles gambiae s.l.* mosquitoes from Mali on *Plasmodium falciparum* malaria transmission

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A naturally occurring *Wolbachia* strain (*w*Anga-Mali) was identified in mosquitoes of the *Anopheles gambiae* complex collected in the Malian villages of Dangassa and Kenieroba. Phylogenetic analysis of the nucleotide sequence of two 16S rRNA regions showed that *w*Anga-Mali clusters with *Wolbachia* strains from supergroup A and has the highest homology to a *Wolbachia* strain isolated from cat fleas (*Ctenocephalides*). *w*Anga-Mali is different from two *Wolbachia* strains previously reported in *A. gambiae* from Burkina Faso (*w*Anga_VK5_STP and *w*Anga_VK5_3.1a). Quantitative analysis of *Wolbachia* and *Plasmodium* sporozoite infection in field-collected mosquitoes indicates that the prevalence and intensity of *Plasmodium falciparum* sporozoite infection is significantly lower in *Wolbachia*-infected females. The presence of *Wolbachia* in females from a laboratory *Anopheles coluzzii* (*A. gambiae*, M form) colony experimentally infected with *P. falciparum* (NF54 strain) gametocyte cultures slightly enhanced oocyst infection. However, *Wolbachia* infection significantly reduced the prevalence and intensity of sporozoite infection, as observed in the field. This indicates that *w*Anga-Mali infection does not limit early stages of *Plasmodium* infection in the mosquito, but it has a strong deleterious effect on sporozoites and reduces malaria transmission.

Wolbachia | *Plasmodium* | mosquito | malaria | *Anopheles*

Despite recent strides in reducing the burden of malaria, this disease was still responsible for more than 400,000 deaths in 2015 (1). Most mortality is caused by *Plasmodium falciparum* infection in children from sub-Saharan Africa, where *Anopheles gambiae* and *Anopheles coluzzii* mosquitoes are the major disease vectors. These two anopheline species are traditionally known as the S and M molecular forms of *A. gambiae*, respectively (2, 3). They share limited genetic flow, occupy distinct ecological niches (4), and were only recently reclassified as different species (5). At present, vector control relies mostly on insecticide-based strategies, such as indoor spraying or long-lasting insecticide-treated nets. However, efficacy concerns and reports of evolving insecticide resistance highlight the need to develop alternative malaria control strategies (6).

Wolbachia is a genus of Gram-negative endosymbiotic proteobacteria that are vertically transmitted and commonly found in nematodes and arthropods (7). Several strains of *Wolbachia* are able to manipulate host reproduction by a mechanism known as cytoplasmic incompatibility (CI) (8, 9), which allows *Wolbachia* to reach high prevalence in natural populations (10, 11). Some strains of *Wolbachia* protect insect hosts from viral infections (12–14). For example, the presence of *Wolbachia* in *Aedes aegypti* mosquitoes prevented laboratory infections with dengue and other flaviviruses (15, 16). Based on these findings, a program to release *Wolbachia*-infected mosquitoes at several different test sites around the world was implemented, with the aim of spreading *Wolbachia*-mediated resistance to viruses in natural mosquito populations (17). *Wolbachia* also reduces mosquito susceptibility to other nonviral pathogens. For example, infection of *Anopheles stephensi* with the *Aedes albopictus* *w*AlbB *Wolbachia* strain reduced *P. falciparum* infection, but the effect was modest (18). Similar attempts to artificially infect *A. gambiae* with *Wolbachia* were limited to somatic tissues (19–21), suggesting that *A. gambiae* is less susceptible to *Wolbachia* infections. More recently, populations of *Wolbachia*-infected *A. gambiae* and *A. coluzzii* were identified in Burkina Faso. This strain was called *w*Anga (22), and mosquitoes positive for *Wolbachia* were reported to have a lower prevalence of *Plasmodium* infection (23). However, because the prevalence of *Plasmodium* infection in field-collected mosquitoes is relatively low (5.4% in this study), the analysis was based on a total of 12 *P. falciparum*-infected females (23).

In the present report, we identified *A. gambiae s.l.* mosquitoes in Mali that are naturally infected with *w*Anga-Mali, a *Wolbachia* strain that is different from the ones reported in Burkina Faso. *w*Anga-Mali was first detected in *A. gambiae* and *A. coluzzii* mosquitoes collected in 2010–2011, and its persistence was confirmed in recent collections in 2015–2016. We investigated the impact of *Wolbachia* on *P. falciparum* infection in the field, by

Significance

The introduction of *Wolbachia* (an intracellular bacterium that does not infect higher organisms) into culicine mosquito populations from endemic areas is a promising strategy to prevent arboviral transmission. Anopheline mosquitoes were thought to be naturally refractory to *Wolbachia*, but a population of *Anopheles gambiae* from Burkina Faso infected with *Wolbachia* was recently reported. We identified a *Wolbachia* strain in *A. gambiae* mosquitoes from Mali (*w*Anga-Mali). *w*Anga-Mali infection was associated with reduced prevalence and intensity of sporozoite infection in field-collected females. Experimental infections indicate that *w*Anga-Mali infection reduces malaria transmission by a mechanism that affects sporozoites and opens the possibility of exploring the introduction of *Wolbachia* into natural populations of anophelines as a strategy to reduce disease transmission.

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Data deposition: The data reported in this paper have been deposited in GenBank, https://www.ncbi.nlm.nih.gov/genbank/ (accession nos. MF946612–MF946614, MF944223, and MF944114).

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analyzing a large number of naturally infected mosquitoes, and also under controlled laboratory conditions, by establishing a *Wolbachia*-infected *A. coluzzii* colony.

Results

Identification of *Wolbachia* in Natural Mosquito Populations from Mali. We first analyzed a collection of 13,321 *A. gambiae s.l.* mosquitoes collected in six Malian villages using the intradomiciliary spray-catch technique (24) (Table S1). The thorax and head region was dissected, homogenized, and part of the sample was used to detect the presence of *P. falciparum* sporozoites using an anticircumsporozoite protein (CSP) in ELISAs (24). A total of 205 females (1.53%) were positive for sporozoite infection. The rest of the homogenate from *Plasmodium*-positive samples was used to extract genomic DNA. A broad screen was carried out to analyze a large number of samples and try to identify a village(s) in which mosquitoes would be infected with *Wolbachia*. To this end, pools of 10 mosquitoes were analyzed for the presence of *Wolbachia* infection using the nested PCR-based assay previously used in Burkina Faso (23). One positive pool was detected in the village of Kenieroba and four in Dangassa. Analysis of individual mosquitoes in the positive pools confirmed that there were five *Wolbachia*-positive mosquitoes (one in each pool). *Wolbachia* was not detected in the pools of *Plasmodium*-infected females from the other four villages. We therefore decided to focus our studies in the villages of Kenieroba and Dangassa (Fig. 1*A*), where the presence of *Wolbachia* and *Plasmodium* coinfections had been confirmed.

Development of a Quantitative Assay for *Wolbachia* Infection. A quantitative PCR (qPCR)-based detection method was developed to establish both the prevalence and the intensity of *Wolbachia* infection in natural mosquito populations, and was compared with two other previously described PCR-based detection methods (23, 25). A set of 69 mosquitoes from Kenieroba and Dangassa that were not infected with *Plasmodium* were analyzed using three different methods to detect *Wolbachia* in the same sample. The 16S ribosomal RNA (rRNA) gene was amplified using regular PCR (W16S-Spec) (25), nested PCR (W16S-WE) (23), and a qPCR assay (W16S-qPCR) (Table S2). As expected, regular PCR was the least sensitive method and detected *Wolbachia* in 16% of the samples (11/69). Using nested PCR, 52% of samples (36/69) were positive in at least one of the technical duplicates. We found that with the regular and nested PCR assays, 22% and 19% of technical duplicates, respectively, were not concordant, suggesting that *Wolbachia* levels in mosquitoes from Mali are close to the limit of detection of these assays. As expected, the qPCR method was more sensitive. It detected *Wolbachia* (W^+) in 67% of mosquitoes (46/69), and the correlation between technical replicates was high ($R^2 = 0.9978$, $P < 0.0001$). Therefore, all other detections of *Wolbachia* in these studies were done using the qPCR method. All of the determinations of *Wolbachia* infection prevalence in these two villages (Fig. 1*A*) were done in females that were negative for *P. falciparum* infection, because they represent the majority of the population (97–99% of females were not infected with *Plasmodium*) and because a biological interaction between *Wolbachia* and *P. falciparum* could greatly bias the prevalence of *Wolbachia* in the *Plasmodium*-infected group. The prevalence of *Wolbachia* in female mosquitoes collected in 2010 from Dangassa (61%) was not significantly different from that in Kenieroba (76%) (Fig. 1*A*). However, in 2015, the prevalence (78%) and intensity of *Wolbachia* infection (Fig. S1) in Kenieroba were both significantly higher than in Dangassa (46%) ($P < 0.00001$, χ^2 and $P < 0.0001$, Mann–Whitney, respectively) (Fig. 1*A*). A recent collection in 2016 confirmed that the prevalence of *Wolbachia* in Kenieroba was still very high (38/40 W^+ females = 95%).

Phylogenetic Analysis of *Wolbachia* in *A. gambiae* Mosquitoes from Mali. A highly conserved region of the 16S rRNA gene was amplified using regular PCR with *Wolbachia*-specific primers (25) (Table S2). Sequencing of the PCR products (accession no. MF944114) confirmed that *Wolbachia* in *A. gambiae* mosquitoes from Mali (*w*Anga-Mali) clusters with *Wolbachia* strains of supergroup A (97–99.8% nucleotide identity) and has lower homology to *Wolbachia* strains of supergroup B (94–95%) and to other closely related bacterial species (*Anaplasma phagocytophilum* 93%; *Ehrlichia chaffeensis* 91%; *Rickettsia japonica* 88%) (Fig. 1*B* and Fig. S2). The sequences of this conserved region are also available for *Wolbachia* strains isolated from *A. gambiae* mosquitoes from Burkina Faso (we will refer to them as *w*Anga-BF) (22). Phylogenetic analysis indicates that one of the reported *w*Anga-BF sequences clusters with *Wolbachia* supergroup A (*w*Anga_VK5_STP) and shares 97% identity with *w*Anga-Mali, while the second strain (*w*Anga_VK5_3.1a) is more divergent, clusters with supergroup B, and has 94% identity with *w*Anga-Mali (Fig. 1*B*). Interestingly, *w*Anga-Mali has the highest homology (99.8%) to *Wolbachia* from cat fleas (*Ctenocephalides*).

We amplified a second region of 16S rRNA that is more variable, using primers that amplify both *Wolbachia* and closely related bacteria (25), which allows for a more detailed comparison between sequences from different *Wolbachia* strains. The *w*Anga-Mali sequence (accession no. MF944223) clustered with *Wolbachia* strains that infect other arthropods, and also had the highest homology to that from cat fleas (*Ctenocephalides*) (Fig. 1*C*). The phylogeny of the different *Wolbachia* strains does not match the phylogeny of their hosts. For example, *w*Anga-Mali is more closely related to *Wolbachia* from cat fleas than to those present in other mosquitoes, such as *w*AlbB (*A. albopictus*) and *w*Pip (*Culex pipiens*), indicating that acquisition of this symbiont occurred by horizontal transfer. *w*Anga-Mali is evolutionarily very distant from *Wolbachia* that infects *Brugia malayi* (Fig. 1*C*), eliminating the possibility that *w*Anga-Mali could be a contamination from mosquitoes infected with this nematode.

The multilocus sequence typing (MLST) scheme is a universal genotyping tool for *Wolbachia* (26) that uses the sequence of specific regions from five conserved genes [*gatB*: aspartyl/glutamyl-tRNA(Gln) amidotransferase, subunit B; *coxA*: cytochrome *c* oxidase, subunit I; *hcpA*: conserved hypothetical protein; *ftsZ*: cell division protein; and *fbpA*: fructose-bisphosphate aldolase] to classify newly identified *Wolbachia* strains. We attempted to characterize *w*Anga-Mali using this system, but we were only able to amplify three of the five genes (accession nos. *hcpA*, MF946614; *fbpA*, MF946613; *coxA*, MF946612) (Figs. S3–S5). Multiple attempts to amplify the two remaining genes (*gatB*, *ftsZ*) were not successful, suggesting there may be some degree of sequence divergence in the primer region. The three amplified regions (from the *hcpA*, *fbpA*, *coxA* genes) all had higher homology to *Wolbachia* (91–93%) than to *Ehrlichia* (56–74%), *Anaplasma* (55–73%), or *Rickettsia* (46–70%) (Table S3). Taken together, these data indicate that *Wolbachia* is present in *A. gambiae* mosquitoes from Mali, and is not identical to *w*Anga-BF strains previously reported in Burkina Faso.

Correlation Between *Wolbachia* and *Plasmodium* Infections in the Field. ELISAs were used to detect the presence of *P. falciparum* sporozoites in homogenates from the thorax and head region. Genomic DNA was extracted from 62 *Plasmodium*-positive and 69 *Plasmodium*-negative females collected in 2010–2011. The relative levels of *Plasmodium* and *Wolbachia* in each sample were determined by quantitative amplification of the 28S and 16S rRNA genes, respectively (Fig. 2*A*). We found that 65/131 (49.6%) of females were infected with *Wolbachia*. It is apparent from the distribution of the data that most values cluster either along the *x* or the *y* axis (Fig. 2*A*). The observed proportion of females coinfected with *Wolbachia* and *Plasmodium* (green dots, Fig. 2*A*) (19/131 = 14.5%) was lower than expected (23.4%) based on the prevalence of *Plasmodium* (47.3%) and *Wolbachia* (49.6%) infection (expected prevalence = 0.473 × 0.496 =0.234), indicating that females infected with one organism are less likely to be infected with the other. The prevalence of *Plasmodium* infection in

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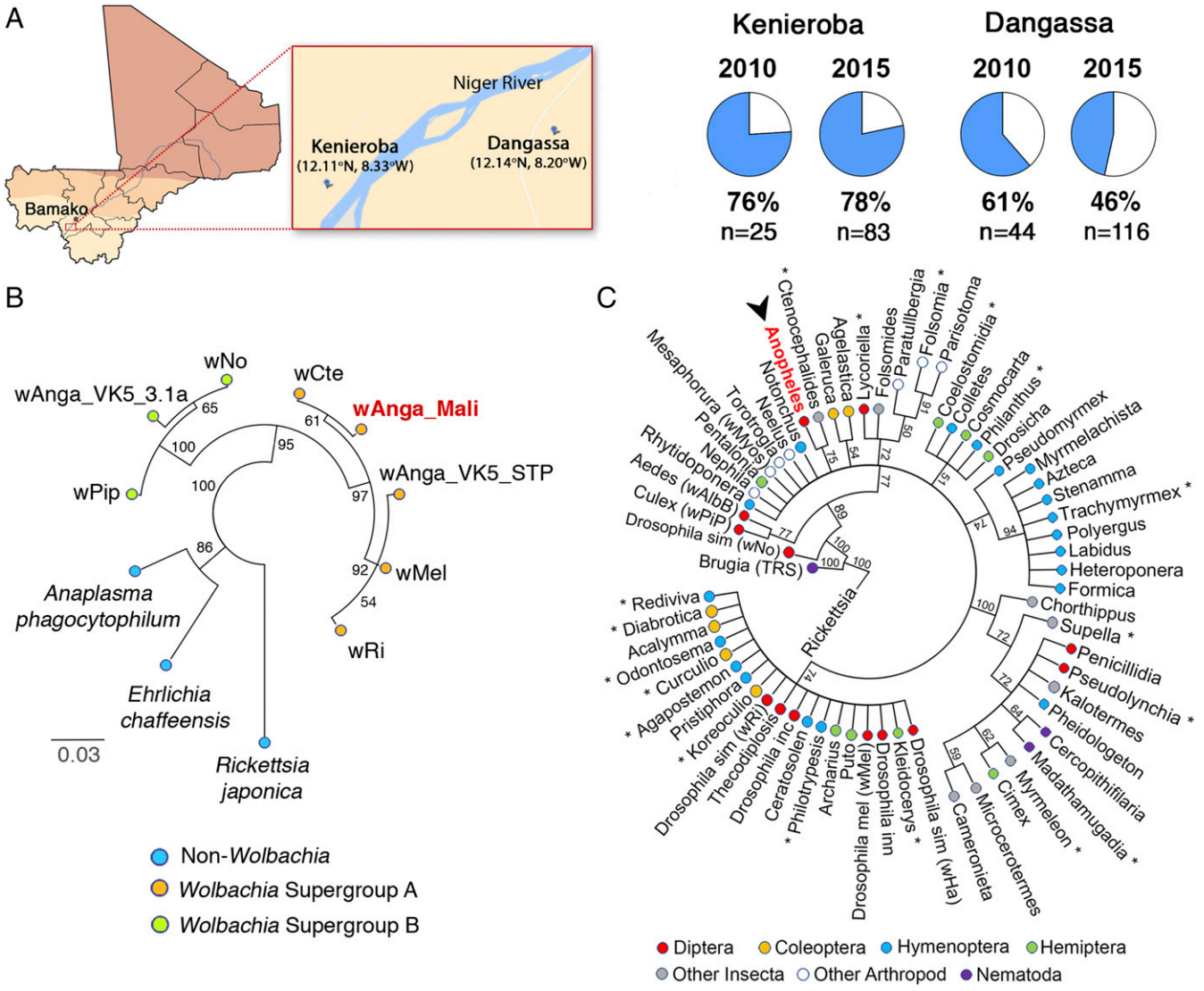

Fig. 1. A *Wolbachia* strain is present in *A. gambiae* and *A. coluzzii* mosquitoes from Mali. (*A*) Map of Mali and geographic localization of the villages of Dangassa and Kenieroba, where *A. gambiae* (S molecular form) and *A. coluzzii* (M molecular form) mosquitoes naturally infected with *Wolbachia* were identified (the geographic coordinates of the villages are indicated). The prevalence of *Wolbachia* in *A. gambiae senso lato* (*s.l.*) (*w*Anga-Mali) infection in samples collected in these two villages, in 2010 and 2015, is also indicated. (*B*) Phylogenetic analysis based on the alignment of a conserved region of the 16S rRNA gene using *Wolbachia*-specific primers. The sequence of *w*Anga-Mali (highlighted in red) clusters with *Wolbachia* strains from the supergroup A has the highest homology from Wolbachia isolated from cat fleas (*Ctenocephalides*) and is different from sequences previously reported for *A. gambiae s.l.* from Burkina Faso (Anga_VK5_STP and wAnga_VK5_3.1a). Sequences from other non-*Wolbachia* proteobacteria were also included, and the sequence from *R. japonica* was used as the reference outgroup. (*C*) Phylogenetic analysis based on the alignment of a variable region of the 16S rRNA gene from *Wolbachia* strains isolated from the nematode *B. malayi*, insects, and other arthropods. The sequence from *R. japonica* was used as the reference outgroup. Strains are identified by their host genus and color-coded according to their taxonomic order. The *w*Anga-Mali strain is shown red and is indicated by the arrowhead. Asterisks (*) denote when the consensus sequence for closely related sequences from the same genus was used. The numbers indicated the consensus support (%).

Wolbachia positive (W^+) females (29%) was significantly lower than that of *Wolbachia* negative (W^-) females (65%, $P < 0.0001$, χ^2) (Fig. 2*B*). The intensity of *Plasmodium* infection was also significantly reduced in W^+ females ($P < 0.001$, Mann–Whitney). Genotyping revealed that 34 females (26%) were S form (*A. gambiae*), while 95 (72%) were M form (*A. coluzzii*). The prevalence of *Wolbachia* infection in *A. gambiae* (53.6%) was not significantly different from that of *A. coluzzii* (41.2%). Similar results were obtained when only *A. coluzzii* females ($n = 95$) were included in the analysis. The prevalence of *Plasmodium* infection in W^+ *A. coluzzii* females (34.2%) was significantly lower than in W^- females (66%) ($P = 0.002$, χ^2), and the intensity of *Plasmodium* infection was also significantly lower ($P = 0.005$) in W^+ females (Fig. S6). These findings from females collected in 2010–2011 were confirmed by analyzing mosquitoes collected in 2015–2016. ELISAs were done in 1,114 *A. gambiae* females, and 23 *Plasmodium*-positive mosquitoes were detected (2.0% infection prevalence). Genomic DNA was extracted from these 23 *Plasmodium*-positive and from 139 *Plasmodium*-negative females, and *Wolbachia* and *Plasmodium* infection levels were determined by qPCR (Fig. 2*C*). The prevalence of *Plasmodium* infection was also significantly lower (8%) in W^+ than in W^- females (26%, $P < 0.01$, χ^2), and the intensity of *Plasmodium* infection was significantly reduced ($P = 0.002$, Mann–Whitney) (Fig. 2*D*). It is worth noting that the W^+ females in the 2015–2016 collection that were infected with *Plasmodium* had low levels of *Wolbachia* infection (Fig. 2*C*). Taken together, these observations are in agreement with the previous report of reduced prevalence of *Plasmodium* infection in

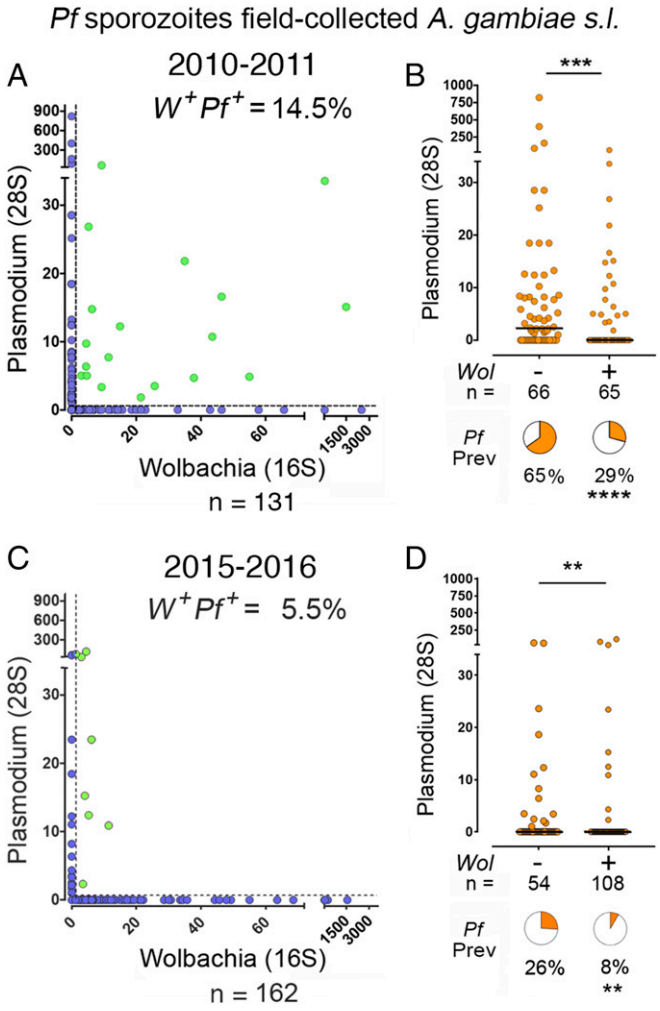

Fig. 2. Effect of naturally occurring *Wolbachia* on *Plasmodium* sporozoite infection in field-collected mosquitoes. *Wolbachia* and *Plasmodium* levels in field-collected mosquitoes were determined by qRT-PCR. (*A* and *C*) Correlation between *Wolbachia* and *Plasmodium* levels. Coinfected mosquitoes (W^+Pf^+) are highlighted in green, and their relative abundance is indicated. (*B* and *D*) 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. 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). $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$.

A. coluzzii females that carry *w*Anga-BF (23) and support the hypothesis that *Wolbachia* infection reduces *Plasmodium* infection in *A. coluzzii*. The sample size for *A. gambiae* S form ($n = 34$ in 2010–2011 and 13 in 2015–2016) was not large enough to carry out an independent analysis.

Laboratory Adaptation of *Wolbachia*-Infected Mosquitoes and Effect of *Wolbachia* on *Plasmodium* Infections. To further evaluate the effect of *Wolbachia* on *Plasmodium* transmission under controlled conditions, a colony of *A. coluzzii* mosquitoes was established from mosquito eggs brought from Mali (Dangassa) in 2015. The first three generations of field-collected mosquitoes were supplemented with male mosquitoes from an *A. coluzzii* colony established at NIH several years ago (*A. gambiae* M-form NIH) that were already adapted to mate in captivity. This colony was also established from eggs collected in Mali, and we confirmed that these mosquitoes were not infected with *Wolbachia*. The *A. gambiae* M-form NIH strain is a reference mosquito strain, and its genome has been sequenced (27). Mosquitoes were infected with *P. falciparum* (NF54 strain) by membrane feeding of gametocyte cultures, and the levels of both *Plasmodium* and *Wolbachia* infection were determined from genomic DNA extracted from midguts collected 8–10 d after infection. We were surprised to find that the observed prevalence of midgut coinfections (40/104 = 38%; Fig. 3*A*, green dots) was higher than expected, based on the prevalence of *Wolbachia* and *Plasmodium* infection (expected prevalence = 33%). Furthermore, opposite to what was observed in the field for sporozoite infections, the prevalence ($P < 0.05$, χ^2) and the intensity of *Plasmodium* oocyst infection ($P < 0.05$, Mann–Whitney) were both moderately, but significantly, higher in

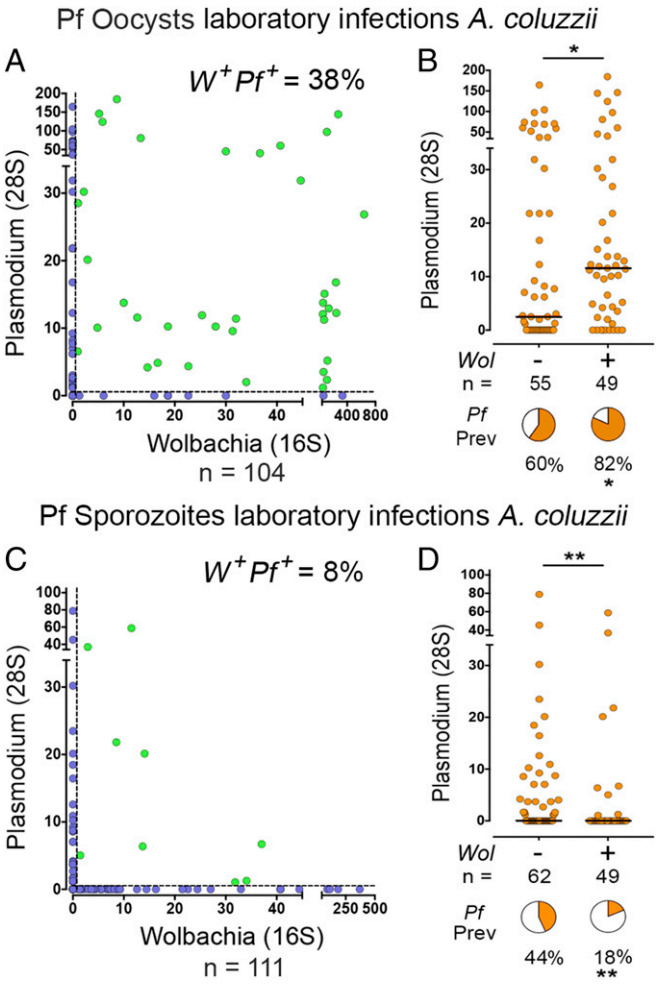

Fig. 3. Effect of *Wolbachia* in laboratory-reared mosquitoes on *Plasmodium* infection. *Wolbachia* and *Plasmodium* levels in laboratory-reared *A. coluzzii* mosquitoes challenged with *P. falciparum* NF54 were determined by qRT-PCR. (*A*) Correlation between midgut *Wolbachia* and *Plasmodium* levels 10 d after infection. Coinfected mosquitoes (W^+Pf^+) are highlighted in green, and their relative abundance is indicated. (*B*) Midgut levels of *Plasmodium* 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. (*C*) Correlation between *Wolbachia* and *Plasmodium* levels 21 d after infection. Coinfected mosquitoes (W^+Pf^+) are highlighted in green, and their relative abundance is indicated. (*D*) Head-thorax 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. Value distributions were compared using the Mann–Whitney test. Pie charts represent *Plasmodium* prevalence in *Wolbachia*-infected and uninfected samples. Prevalence was compared using the χ^2 test (n = number of samples). $^{*}P < 0.05$, $^{**}P < 0.01$.

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females that carried *Wolbachia* infections. These data indicate that *Wolbachia* midgut infection does not negatively impact the early stages of *Plasmodium* development in the mosquito.

Because the field studies were done by detecting sporozoite infection in the salivary glands, the effect of *Wolbachia* on this later stage of the parasite was also evaluated. *Wolbachia* and *Plasmodium* levels were analyzed in thorax and head samples collected 18–21 d after infection. Similar to what was observed in the field, the observed prevalence of coinfection was lower than expected (9/111 = 8%; Fig. 3*C*, green dots) based on the prevalence of *Wolbachia* and *Plasmodium* infections (expected prevalence = 14.3%). The prevalence of *Plasmodium* infection in W^+ females (18%) was significantly lower ($P < 0.004$, χ^2) than that of W^- females (44%) (Fig. 3*D*). The intensity of *Plasmodium* infection was also significantly reduced ($P < 0.01$, Mann–Whitney). Taken together, these findings show that although *Plasmodium* oocyst infections were slightly higher in *Wolbachia*-infected females, the number of sporozoites was significantly reduced.

Discussion

Wolbachia is widely prevalent among arthropods and is thought to be naturally present in as many as 65% of insect species (28). Other species can be infected under laboratory conditions (29–31). Interestingly, anophelines display a remarkable degree of refractoriness to *Wolbachia*. A stable infection of *A. stephensi* with *w*AlbB has been established (18), but all other laboratory infections of anophelines have been limited to somatic tissues, and failed to infect the germ line and did not propagate to the offspring (19–21). Field populations of anophelines were thought to be resistant to *Wolbachia* infections. However, *A. gambiae* populations from Burkina Faso naturally infected with *Wolbachia* were recently reported (22). It is not clear whether some *Wolbachia* strains evolved specific adaptations that allowed them to colonize mosquitoes of the *A. gambiae* complex in West Africa, or whether genetic differences already present in West African anophelines favored *Wolbachia* invasion. The observation that two independent PCR amplifications with standard primers from the MLST universal genotyping tool (*gatB*, *ftsZ*) failed in *w*Anga-Mali–infected mosquitoes suggests that *w*Anga-Mali is more divergent in these regions than in other *Wolbachia* strains. The prevalence and distribution, as well as seasonal fluctuations of *Wolbachia* infections in natural anopheline mosquito populations in Africa and other continents, remain to be established.

We investigated whether the presence of *Wolbachia* was a rare local event, limited to Burkina Faso, or a common occurrence in West Africa. We first identified natural populations of *A. gambiae* and *A. coluzzii* infected with *Wolbachia* in the Malian villages of Kenieroba and Dangassa during the wet season of 2010; recent collections from the same villages, in 2015–2016, confirmed that *Wolbachia* is still circulating. The levels of *w*Anga-Mali we detected in anopheline mosquitoes are very low and close to the detection limit of PCR-based assays. For comparison, the copies of the 16S rRNA gene from *w*Anga-Mali that we detect are usually less than 1% of total mosquito genome copies of S7 rRNA. This is a remarkable difference from what has been reported in field-released *w*Mel-infected *A. aegypti* (32), or in *w*AlbB infections of *A. albopictus* (33) or *A. stephensi* (18), where *Wolbachia* genome copies are close to a 1:1 ratio with mosquito genome copies. Phylogenetic analysis of two different regions of 16S rRNA indicates that *w*Anga-Mali is more similar to a *Wolbachia* strain isolated from cat fleas (*Ctenocephalides*) (Fig. 1 *B* and *C*) (99.8% identity) than to the two strains isolated from *A. gambiae* complex mosquitoes in Burkina Faso (94 and 97% identity) (Fig. 1*B*), suggesting that the acquisition of *Wolbachia* in Burkina Faso and Mali were independent events. Interestingly, it was reported that amplification of *fbpA* from the *w*Anga-BF strain failed (22), while we successfully amplified this gene in *w*Anga-Mali using the same primers, further suggesting some sequence divergence between *w*Anga-Mali and *w*Anga-BF. A detailed comparison of the variable region of the 16S rRNA with the isolates from Burkina Faso, as well as whole-genome sequencing, would be necessary to obtain a clear picture of the origin and spread of *Wolbachia* in natural mosquito populations in Africa.

Wolbachia infections of culicine mosquitoes and other dipterans were shown to be protective against several viral pathogens and to reduce *Plasmodium gallinaceum* infection in *A. aegypti* (12–14, 16). Somatic *Wolbachia* infections of anophelines have further suggested that *Wolbachia* could also confer partial protection of mosquitoes against *Plasmodium* (19). Furthermore, a lower *Plasmodium* prevalence was observed in mosquitoes carrying PCR-detectable levels of *Wolbachia* in Burkina Faso. A total of 221 blood-fed *A. coluzzii* obtained from homes were analyzed 5 d after collection. There were 12 infected females, one in the group infected with *Wolbachia* (1/116 = 0.8%) and 11 in those mosquitoes that did not carry *Wolbachia* (11/105 = 10.4%). The detection of the parasite was done using a PCR assay, so it is not clear whether early oocysts or sporozoites from a previous infection were detected. In the present study, we increased the number of infected mosquitoes by prescreening a large sample set using a CSP-based ELISA in homogenates from the thorax and head region to detect sporozoites. We also found a strong negative association between *Wolbachia* infection and the prevalence of *P. falciparum* sporozoite infection ($P < 0.0001$, χ^2). The effect on *A. coluzzii* is clear, but the sample size of *A. gambiae* (S form) was too small to be analyzed independently. Although *Wolbachia* infections have a similar prevalence in *A. coluzzii* and *A. gambiae*, it is not clear whether the same negative correlation with *Plasmodium* infection will also be observed in *A. gambiae* (S form).

Environmental factors and host age affect both *Wolbachia* levels and its effects on host immunity (34–37), and neither factor can be controlled in field-collected mosquitoes. To control such variables, we established a colony of *A. coluzzii* mosquitoes from eggs brought from Dangassa, Mali, in 2015. Infections with the African NF54 strain of *P. falciparum* confirmed that *Wolbachia* negatively impacts sporozoite development. Interestingly, this negative effect was not observed up to the oocyst stage. Furthermore, the effect was opposite, with a modest but significant increase in midgut infection, suggesting that parasites are protected from the effect of *Wolbachia* while inside the oocysts. Perhaps *Wolbachia* infection depletes some nutrients, such as membrane lipids, required by sporozoites, or may activate a stronger immune response that targets sporozoites when they are released into the hemolymph. Alternatively, sporozoites may be damaged in the salivary gland, either during the traversal, as they come in direct contact with the cell cytoplasm, or as they accumulate in the secretory cavity where they are constantly bathed by salivary gland secretions. Stable *w*AlbB infection in *A. stephensi* significantly reduced the number of oocysts, but had a much stronger effect on salivary gland sporozoites (18). The mechanism by which *Wolbachia* infection affects sporozoites remains to be elucidated.

Release of *Wolbachia*-infected *A. aegypti* is one of the most promising strategies to prevent transmission of dengue and other flaviviruses in endemic areas. The rapid success of this intervention is dependent on CI triggered by *Wolbachia* infection, a natural mechanism that drives this bacterium into natural insect populations. In brief, CI is defined as offspring mortality when *Wolbachia*-infected males mate with females that are not infected. Mortality does not occur when infected males mate with females that are also infected. CI gives *Wolbachia*-infected females a reproductive advantage that becomes more effective as the prevalence of *Wolbachia* infection increases in the population, allowing these bacteria to reach very high frequencies within a few generations. However, CI is not found in every *Wolbachia* strain. The *w*Anga-BF strain did not induce CI under laboratory conditions (23). We did not directly evaluate the presence of CI in our colony, but the prevalence of *Wolbachia* infection did not increase during the time of this study (5–15 generations), as would be expected if CI was taking place. CI-associated genes were recently identified in *Drosophila* (38, 39), but nongenetic factors also seem to affect the manifestation of CI (37, 40, 41). In general, the levels

of infection present in mosquitoes from Mali are very low, and the mechanisms or interactions with the mosquito immune system or with other symbionts that may limit *Wolbachia* infection are unknown. Adaptation to tissue culture and genetic analysis of *w*Anga strains will be paramount to analyze whether the genes responsible for CI are present in *Wolbachia* strains circulating in mosquitoes from West Africa.

Collectively, our data support the hypothesis that *Wolbachia* infection, even at low levels, negatively impacts *Plasmodium* sporozoite infection in *A. coluzzii*. In theory, the release of *Wolbachia*-infected mosquitoes could be a promising strategy to reduce transmission, but the lack of clear CI could be an important limitation. It might be feasible, however, to develop a genetically modified *Wolbachia* to induce CI, or to select *Wolbachia* strains that can spread efficiently in natural *A. gambiae* populations. The high prevalence (78%) and increased intensity of *Wolbachia* infection in the village of Kenieroba during the 2015 and 2016 collections, that was not observed in Dangassa, is very interesting and warrants further investigation. This observation suggests that there may be local differences in the adaptation of *Wolbachia* to mosquitoes between these two villages, and/or differences in the time when these bacteria were introduced. It also indicates that, under some conditions, *Wolbachia* infection can reach a high prevalence and that infection levels in the population can increase substantially over time, to levels that could disrupt *P. falciparum* malaria transmission.

Materials and Methods

Intradomiciliary collections *A. gambiae* females were done in several Malian Villages using the spray-catch technique, and mosquitoes were screened for the presence of *P. falciparum* sporozoites using ELISAs and for *Wolbachia* infections using nested PCR. Genomic DNA was extracted from a similar number of females that were either positive or negative for *Plasmodium* infection, and the prevalence and intensity of *Wolbachia* and *P. falciparum* sporozoite infections were determined by qPCR. A laboratory colony of *A. gambiae* M-form (*A. coluzzi*) was established, and the effect of *Wolbachia* infection on the prevalence and intensity of midgut oocysts and salivary gland *P. falciparum* infection was evaluated. Detailed information on the methodology used can be found in *SI Materials and Methods*.

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