Infection of anopheline mosquitoes with *Wolbachia*: Implications for malaria control

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**Malaria eradication and vector control in Africa**

Africa carries a disproportionate share of the malaria burden. For example, more than 90% of the 446,000 malaria-related deaths reported in 2015 occurred in sub-Saharan Africa [1]. *Anopheles gambiae*, *A. coluzzii*, *A. arabiensis*, and *A. funestus* are some of the most important African vectors of malaria [2,3]. Other species, such as *A. melas* and *A. merus* are also efficient vectors but have a limited geographical distribution [3]. Insecticide-based strategies, mainly the distribution of insecticide-treated nets and indoor residual spraying, are efficient against a wide range of mosquitoes and are the current cornerstones of malaria control programs. However, the growing number of reports of insecticide resistance is driving the development of novel vector control strategies [4].

**Wolbachia infection in Drosophila and protection against viruses**

*Wolbachia* is a genus of vertically transmitted endosymbiotic alphaproteobacteria that infect about 40% of arthropod species [5]. *Wolbachia* infection often reaches a high prevalence in natural insect populations, often through the induction of cytoplasmic incompatibility (CI) [6]. Briefly, CI is a mechanism by which the sperm of *Wolbachia*-infected males is unable to form viable offspring when eggs of uninfected females are fertilized, whereas eggs of infected females are viable. This mechanism allows the spread of *Wolbachia* through the population by giving infected females a reproductive advantage.

Many *Wolbachia* strains are known to confer resistance against viral infections. For example, the *Wolbachia* wMel strain protects *Drosophila melanogaster* against *Drosophila* C virus (DCV) [7] and Flock House virus [8], suggesting that protection is efficient against a variety of RNA viruses. Although the molecular basis of protection remains under debate, the *Wolbachia* genome has been shown to be a key player. Protection by genetic variants of wMel *D. melanogaster* correlates with the phylogeny of the *Wolbachia* strains analyzed [9]. Furthermore, viral protection was observed in *D. simulans* lines infected with *Wolbachia* strains phylogenetically related to wMel but not with more divergent strains [10].

**Wolbachia in mosquito vectors and its effects on disease transmission**

*Wolbachia* also protects mosquitoes from viral infections. Native wPip infections in *Culex quinquefasciatus* increases host resistance to West Nile virus [11]. Similarly, native *Wolbachia* limits dengue virus (DENV) infection in *Aedes albopictus* [12]. However, the effect of
Wolbachia on viruses is strain and host specific. For example, native Wolbachia had no effect on DENV infection in Aedes notoscriptus [13].

Transinfection of host with Wolbachia triggers stronger antiviral protection than native Wolbachia strains [14]. The Drosophila wMel-Pop [15] and wMel [16] Wolbachia strains were adapted to infect Aedes aegypti cell lines and were used to transinfect A. aegypti mosquitoes by embryonic microinjection. For both strains, transinfected mosquitoes displayed strong vertical transmission with CI and greatly reduced DENV transmission [16]. A. aegypti transinfected with wMel were released in test sites in Australia, where they replaced natural mosquito populations and reached near fixation levels within a few months after their initial release [17]. Wolbachia prevalence has been stable for several years in these locations [17,18] and has slowly spread throughout the area [19].

A systematic survey in Thailand detected native Wolbachia infections in 23 species of mosquitoes, including species from the genera Aedes, Culex, and Mansonia [20]. None of the 19 species of Anopheles screened were found to harbor Wolbachia. The lack of Wolbachia infections in anophelines was later confirmed in screenings using European, African, and American specimens [21,22].

Thoracic microinjections of Wolbachia in A. gambiae resulted in ubiquitous infections in somatic tissues. However, germline cells were not infected and this precluded vertical transmission [23]. A stable Anopheles stephensi line infected with A. albopictus wAlbB strain was established by embryonic microinjection of A. albopictus ooplasms [24]. Wolbachia-infected A. stephensi were partially protected against Plasmodium falciparum infections, resulting in a modest decrease in oocyst numbers and a strong reduction in salivary gland sporozoites. Similar protection against Plasmodium was also observed when wMel-infected A. aegypti were challenged with P. gallinaceum [15] or when A. gambiae carrying somatic Wolbachia infections were infected with P. falciparum [23]. Other reports have suggested that some combinations of Wolbachia, host, and environmental factors could actually enhance Plasmodium infection in mosquitoes [25,26].

Identification of Wolbachia in natural African anopheline mosquito populations

Recently, traces of Wolbachia genomic DNA were identified in a microbiome survey of the reproductive organs of A. gambiae and A. coluzzii in malaria-endemic areas of Burkina Faso in West Africa (Fig 1A) [27,28]. Wolbachia-specific PCR amplification and sequencing was used to confirm the presence of Wolbachia in these mosquito populations [27,29]. In an independent study, native Wolbachia infections were identified in the same mosquito species collected in two villages from Mali (Fig 1A) [30]. Wolbachia infection was observed in two collections made five years apart, indicating that the symbiosis has remained stable in the population. More recently, native Wolbachia infections were identified in a broad range of African anophelines from the Democratic Republic of Congo, Guinea, Uganda, and Madagascar [31], as well as Gabon [32]. Phylogenetic analysis suggested that several independent horizontal transfers of Wolbachia infection have occurred, but whole-genome sequencing will be necessary for an in-depth analysis of the evolutionary relationship between these strains.

A significant reduction in Plasmodium prevalence was observed in mosquitoes carrying native Wolbachia infection in Mali. This effect was dose dependent, with a nonlinear negative correlation (Fig 1B) [29,30]. Because confounding environmental or ecological variables could influence the analysis of field-collected mosquitoes, a colony of wAnga-Mali-infected A. coluzzii was established. Infection of colony-adapted mosquitoes with P. falciparum NF54...
confirmed that Wolbachia negatively affects sporozoite infection with a similar negative correlation (Fig 1B) [30].

Challenges for the development of Wolbachia as a tool for malaria control

The identification of native Wolbachia infections in A. gambiae that reduce malaria transmission is a remarkable finding. The fact that several species of Anopheles, including all the major malaria vectors in Africa, have been shown to harbor a variety of Wolbachia strains opens the possibility that one of these strains may confer CI and disrupt disease transmission. However, several challenges remain before Wolbachia can be proposed as a tool for malaria control. Implementation of Wolbachia-based strategies would rely on CI for Wolbachia to spread rapidly in natural populations following mosquito releases. At present, it is not clear whether native Wolbachia can induce CI in Anopheles. Induction of CI was not observed in caged experiments using wAnga-BF-infected Anopheles. However, CI has been shown to be influenced by environmental factors [33], and optimal conditions might be different from the ones typically used for rearing laboratory mosquitoes.

Wolbachia levels have also been shown to influence CI [34,35], and the lack of CI might be due to the low levels of native Wolbachia infection in Anopheles (wAnga-Mali genome copies are usually less than 0.1% of the mosquito genome). Wolbachia levels are also positively correlated with the intensity of protection against viruses. Similarly, higher Wolbachia levels may induce CI in anophelines and confer stronger protection against Plasmodium, which will be essential for Wolbachia to be developed as a tool against malaria.
The mechanisms limiting Wolbachia levels are not well understood. One hypothesis is that Wolbachia might have adapted to control its replication as a strategy to hide from host immunity. Alternatively, high levels of Wolbachia could reduce mosquito fitness and be negatively selected. Another possibility is that Anopheles might not be a good host for Wolbachia. For example, the mosquito microbiota limits Wolbachia infections in A. gambiae. More specifically, the presence of bacteria from the genus Asaia in germline cells prevents Wolbachia invasion of A. gambiae ovaries [36]. Microbiome analyses of mosquitoes collected from Wolbachia-endemic areas found that the prevalence of Asaia was lower than in reports from other locations in Africa [28] or failed to find evidence of co-infections between Wolbachia and Asaia [31]. However, the level of P. falciparum infection was not significantly different between females with or without Asaia in A. gambiae s.l. females collected in Guinea [31]. It is also possible that Anopheles metabolism or immunity might limit Wolbachia levels. In Aedes, Wolbachia manipulates host lipid metabolism, and cholesterol sequestration seems to play a role in the protection against viruses [37,38]. The current understanding of Anopheles lipid metabolism is incipient, but poor nutritional stores could explain the inability of anophelines to sustain high Wolbachia densities.

Infection of embryo-derived somatic cell lines would be an invaluable in vitro model to identify the components limiting wAnga levels. Transcriptomics and metabolomics of infected cells would also allow for a detailed analysis of the effect of Wolbachia on host homeostasis. Once potential target genes are identified, it would be possible to carry out functional screens to identify genes or nutrients limiting Wolbachia infection. However, it will still be necessary to evaluate how this information can be translated to a whole mosquito and eventually to the field. An alternative approach would be the development of directed evolution protocols that would allow progressive adaptation of Wolbachia or hosts to sustain higher bacteria levels.

**Future perspective**

Laboratory transinfection of Wolbachia to African vectors of malaria has been limited to somatic tissues, and Wolbachia failed to be vertically transmitted. We now know that Wolbachia strains in Africa have been able to overcome this barrier and establish infections in anophelines. The presence of this bacteria negatively correlates with Plasmodium sporozoite prevalence. This is an exciting new development in the field, but several challenges remain. It is not clear whether those strains can induce CI, which is needed for implementation of Wolbachia as a control strategy. Further studies should address this question and identify the factors limiting Wolbachia replication in anophelines. A more detailed understanding of the molecular components mediating Wolbachia establishment and host adaptation might make it possible to adapt CI-carrying Wolbachia strains to infect anophelines or to increase the levels of native Wolbachia infections and disrupt malaria transmission.

**References**

1. World Health Organization. World Malaria Report 2015. World Health Organization; 2016.
2. Sinka ME, Bangs MJ, Manguin S, Rubio-Palis Y, Chareonviriyaphap T, Coetzee M, et al. A global map of dominant malaria vectors. Parasit Vectors. BioMed Central; 2012; 5: 69.
3. Wiebe A, Longbottom J, Gleave K, Shearer FM, Sinka ME, Massey NC, et al. Geographical distributions of African malaria vector sibling species and evidence for insecticide resistance. Malar J. BioMed Central; 2017; 16: 85.
4. Ranson H, N’guessan R, Lines J, Moiroux N, Nkuni Z, Corbel V. Pyrethroid resistance in African anopheline mosquitoes: what are the implications for malaria control? Trends Parasitol. 2011; 27: 91–98. https://doi.org/10.1016/j.pt.2010.08.004 PMID: 20843745
5. Zug R, Hammerstein P. Still a host of hosts for Wolbachia: analysis of recent data suggests that 40% of terrestrial arthropod species are infected. PLoS ONE. 2012; 7: e38544. https://doi.org/10.1371/journal.pone.0038544 PMID: 22685581

6. Werren JH, Baldo L, Clark ME. Wolbachia: master manipulators of invertebrate biology. Nat Rev Microbiol. 2008; 6: 741–751. https://doi.org/10.1038/nrmicro1969 PMID: 18794912

7. Hedges LM, Brownlie JC, O'Neill SL, Johnson KN. Wolbachia and Virus Protection in Insects. Science. 2008; 322: 702–702. https://doi.org/10.1126/science.1162418 PMID: 18974344

8. Teixeira L, Ferreira A, Ashburner M. The bacterial symbiont Wolbachia induces resistance to RNA viral infections in Drosophila melanogaster. PLoS Biol. 2008; 6: e2.

9. Chrostek E, Marialva MSP, Esteves SS, Weinert LA, Martinez J, Jiggins FM, et al. Wolbachia variants induce different protection to viruses in Drosophila melanogaster: a phenotypic and phylogenomic analysis. PLoS Genet. 2013; 9: e1003896. https://doi.org/10.1371/journal.pgen.1003896 PMID: 24348259

10. Osborne SE, Leong YS, O'Neill SL, Johnson KN. Variation in antiviral protection mediated by different Wolbachia strains in Drosophila simulans. PLoS Pathog. 2009; 5: e1000656. https://doi.org/10.1371/journal.ppat.1000656 PMID: 19911047

11. Glaser RL, Meola MA. The native Wolbachia endosymbionts of Drosophila melanogaster and Culex quinquefasciatus increase host resistance to West Nile virus infection. PLoS ONE. 2010; 5: e11977. https://doi.org/10.1371/journal.pone.0011977 PMID: 20700535

12. Mousson L, Zouache K, Arias-Goe ta C, Raquin V, Mavingui P, Failloux A-B. The native Wolbachia symbionts limit transmission of dengue virus in Aedes albopictus. PLoS Negl Trop Dis. 2012; 6: e1089. https://doi.org/10.1371/journal.pntd.0001989 PMID: 23301109

13. Skelton E, Rances E, Frentiu FD, Kusmintarsih ES, Iturbe-Ormaetxe I, Caragata EP, et al. A native Wolbachia Endosymbiont does not limit dengue virus infection in the mosquito Aedes notoscriptus (Diptera: Culicidae). J Med Entomol. Oxford University Press; 2015; 53: 401–408.

14. Bian G, Zhou G, Lu P, Xi Z. Replacing a native Wolbachia with a novel strain results in an increase in endosymbiont load and resistance to dengue virus in a mosquito vector. PLoS Negl Trop Dis. 2013; 7: e2250. https://doi.org/10.1371/journal.pntd.0002250 PMID: 23755311

15. Moreira LA, Iturbe-Ormaetxe I, Jeffery JA, Lu G, Pyke AT, Hedges LM, et al. A Wolbachia symbiont in Aedes aegypti limits infection with dengue, Chikungunya, and Plasmodium. Cell. 2009; 139: 1268–1278. https://doi.org/10.1016/j.cell.2009.11.042 PMID: 20064373

16. Walker T, Johnson PH, Moreira LA, Iturbe-Ormaetxe I, Frentiu FD, McMeniman CJ, et al. The wMel Wolbachia strain blocks dengue and invades caged Aedes aegypti populations. Nature. 2011; 476: 450–453. https://doi.org/10.1038/nature10355 PMID: 21866159

17. Hoffmann AA, Montgomery BL, Popovic J, Iturbe-Ormaetxe I, Johnson PH, Muzzi F, et al. Successful establishment of Wolbachia in Aedes populations to suppress dengue transmission. Nature. 2011; 476: 454–457. https://doi.org/10.1038/nature10356 PMID: 21866160

18. Hoffmann AA, Iturbe-Ormaetxe I, Callahan AG, Phillips BL, Billington K, Axford JK, et al. Stability of the wMel Wolbachia Infection following Invasion into Aedes aegypti Populations. PLoS Negl Trop Dis. 2014; https://doi.org/10.1371/journal.pntd.0003115 PMID: 25211492

19. Schmidt TL, Barton NH, Rasˇić G, Turley AP, Montgomery BL, Iturbe-Ormaetxe I, et al. Local introduction and heterogeneous spatial spread of dengue-suppressing Wolbachia through an urban population of Aedes aegypti. Read A, editor. PLoS Biol. CUP; 2017; 15: e2001894. https://doi.org/10.1371/journal.pbio.2001894 PMID: 28557993

20. Kittayapong P, Baisley KJ, Baimai V, O'Neill SL. Distribution and diversity of Wolbachia infections in Southeast Asian mosquitoes (Diptera: Culicidae). J Med Entomol. 2000; 37: 340–345. PMID: 15535575

21. Rasgon JL, Scott TW. An initial survey for Wolbachia (Rickettsiales: Rickettsiaceae) infections in selected California mosquitoes (Diptera: Culicidae). J Med Entomol. 2004; 41: 255–257. PMID: 15061286

22. Ricci I, Cancrini G, Gabrielli S, D’Amelio S, Favi G. Searching for Wolbachia (Rickettsiales: Rickettssiaceae) in mosquitoes (Diptera: Culicidae): large polymerase chain reaction survey and new identifications. J Med Entomol. 2002; 39: 562–567. PMID: 12144285

23. Hughes GL, Koga R, Xue P, Fukatsu T, Rasgon JL. Wolbachia infections are virulent and inhibit the human malaria parasite Plasmodium falciparum in Anopheles gambiae. PLoS Pathog. 2011; 7: e1002043. https://doi.org/10.1371/journal.ppat.1002043 PMID: 21625582

24. Yin G, Joshi D, Dong Y, Lu P, Zhou G, Pan X, et al. Wolbachia invades Anopheles stephensi populations and induces refractoriness to Plasmodium infection. Science. 2013; 340: 748–751. https://doi.org/10.1126/science.1236192 PMID: 23661760
25. Hughes GL, Vega-Rodriguez J, Xue P, Rasgon JL. Wolbachia strain wAlbB enhances infection by the rodent malaria parasite Plasmodium berghei in Anopheles gambiae mosquitoes. Appl Environ Microbiol. 2012; 78: 1491–1495. https://doi.org/10.1128/AEM.06751-11 PMID: 22210220

26. Murdock CC, Blanford S, Hughes GL, Rasgon JL, Thomas MB. Temperature alters Plasmodium blocking by Wolbachia. Sci Rep. 2014; 4: 3932. https://doi.org/10.1038/srep03932 PMID: 24488176

27. Baldini F, Segata N, Pompon J, Marcenac P, Shaw WR, Dabiré RK, et al. Evidence of natural Wolbachia infections in field populations of Anopheles gambiae. Nat Commun. 2014; 5: 3985. https://doi.org/10.1038/ncomms4985 PMID: 24905191

28. Segata N, Baldini F, Pompon J, Garrett WS, Truong DT, Dabiré RK, et al. Temperature alters Plasmodium blocking by Wolbachia. Sci Rep. 2014; 4: 3932. https://doi.org/10.1038/srep03932 PMID: 24488176

29. Shaw WR, Marcenac P, Childs LM, Buckee CO, Baldini F, Sawadogo SP, et al. Wolbachia infections in natural Anopheles populations affect egg laying and negatively correlate with Plasmodium development. Nat Commun. 2016; 7: 11772. https://doi.org/10.1038/ncomms11772 PMID: 27243367

30. Gomes FM, Hixson BL, Tyner MDW, Ramirez JL, Canepa GE, e Silva TLA, et al. Effect of naturally occurring Wolbachia in Anopheles gambiae mosquitoes from Mali on Plasmodium falciparum malaria transmission. Proceedings of the National Academy of Sciences. National Acad Sciences; 2017; 201716181.

31. Jeffries CL, Lawrence GG, Golovko G, Kristan M, Osborne J, Spence K, et al. Novel Wolbachia strains in Anopheles malaria vectors from Sub-Saharan Africa [Internet]. bioRxiv. 2018. p. 338434. https://doi.org/10.1101/338434

32. Ayala D, Akone-Ella O, Rahola N, Kengne P, Mezeme F, et al. Natural Wolbachia infections are common in the major malaria vectors in Central Africa [Internet]. bioRxiv. 2018. p. 343715. https://doi.org/10.1101/343715

33. Clancy DJ, Hoffmann AA. Environmental effects on cytoplasmic incompatibility and bacterial load in Wolbachia-infected Drosophila simulans. Entomol Exp Appl. Wiley Online Library; 1998; 86: 13–24.

34. Breeuwer JA, Warren JH. Cytoplasmic incompatibility and bacterial density in Nasonia vitripennis. Genetics. 1993; 135: 565–574. PMID: 8244014

35. Calvitti M, Marini F, Desiderio A, Puggioli A, Moretti R. Wolbachia density and cytoplasmic incompatibility in Aedes albopictus: concerns with using artificial Wolbachia infection as a vector suppression tool. PLoS ONE. 2015; 10: e0121813. https://doi.org/10.1371/journal.pone.0121813 PMID: 25812130

36. Hughes GL, Dodson BL, Johnson RM, Murdock CC, Tsujimoto H, Suzuki Y, et al. Native microbiome impedes vertical transmission of Wolbachia in Anopheles mosquitoes. Proc Natl Acad Sci U S A. 2014; 111: 12498–12503. https://doi.org/10.1073/pnas.1408881111 PMID: 25114252

37. Geoghegan V, Stainton K, Rainey SM, Ant TH, Dowie AA, Larson T, et al. Perturbed cholesterol and vesicular trafficking associated with dengue blocking in Wolbachia-infected Aedes aegypti cells. Nat Commun. 2017; 8: 526. https://doi.org/10.1038/s41467-017-00610-8 PMID: 28904344

38. Caragata EP, Rancés E, Hedges LM, Goffton AW, Johnson KN, O’Neill SL, et al. Dietary Cholesterol Modulates Pathogen Blocking by Wolbachia. Vernick KD, editor. PLoS Pathog. Public Library of Science; 2013; 9: e1003459. https://doi.org/10.1371/journal.ppat.1003459 PMID: 23825950