Can *Wolbachia* be used to control malaria?

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Malaria is a mosquito-borne infectious disease caused by Plasmodium *parasites transmitted by the infectious bite of* Anopheles *mosquitoes. Vector control of malaria has predominantly focused on targeting the adult mosquito through insecticides and bed nets. However, current vector control methods are often not sustainable for long periods so alternative methods are needed. A novel biocontrol approach for mosquito-borne diseases has recently been proposed, it uses maternally inherited endosymbiotic* Wolbachia *bacteria transinfected into mosquitoes in order to interfere with pathogen transmission. Transinfected* Wolbachia *strains in* Aedes aegypti *mosquitoes, the primary vector of dengue fever, directly inhibit pathogen replication, including* Plasmodium gallinaceum*, and also affect mosquito reproduction to allow* Wolbachia *to spread through mosquito populations. In addition, transient* Wolbachia *infections in* Anopheles gambiae *significantly reduce* Plasmodium *levels. Here we review the prospects of using a* Wolbachia*-based approach to reduce human malaria transmission through transinfection of* Anopheles *mosquitoes.*

Key words: malaria - *Anopheles* - *Plasmodium* - *Wolbachia* - transinfection

Novel methods of malaria vector control - Malaria is a mosquito-borne infectious disease caused by *Plasmodium* parasites transmitted by the bite of infected *Anopheles* mosquitoes. The disease is responsible for approximately 800,000 deaths each year with more than 200 million annual cases (WHO 2010). Vector control for malaria has focused primarily on the adult mosquito through insecticides and bednets. Residual house spraying with chemical insecticides such as DDT has been used but is logistically and economically demanding in many developing countries (Curtis & Mnzava 2000). When used widely in a community, insecticide treated nets treated with pyrethroid insecticides such as permethrin can provide a safe and simple method of control against night biting *Anopheles* mosquitoes (Hawley et al. 2003). However, both methods are dependent on a single class of insecticides (pyrethroids) leading to widespread development of insecticide-resistance (WHO 2010). As these current methods of malaria control may not be sustainable for long periods due to the increase in insecticide resistance and environmental concerns, evaluation of novel control strategies need to be undertaken. A novel biocontrol approach has been proposed recently that uses maternally inherited endosymbiotic *Wolbachia* bacteria transinfected into mosquitoes in order to interfere with pathogen transmission.

Wolbachia's phenotypic effects in insect hosts - *Wolbachia* are Gram-negative, intracellular, endosymbiotic bacteria that manipulate host reproduction to enhance their vertical transmission (Sinkins et al. 1997). *Wolbachia* bacteria were first reported within the reproductive tissues of *Culex pipiens* mosquitoes by Hertig and Wolbach in 1924 and the species was named *Wolbachia pipientis* (Werren 1997). Infections are extremely widespread within arthropods (Werren et al. 1995, O'Neill et al. 1997, Jeyaprakash & Hoy 2000) and *Wolbachia* are present in numerous mosquito genera including *Aedes*, *Culex*, *Coquillettidia* and *Mansonia* (Kittayapong et al. 2000, Ricci et al. 2002, Dean & Dobson 2004, Tsai et al. 2006). *Wolbachia* are maternally transmitted through the egg cytoplasm and are responsible for several reproductive disorders in their insect hosts such as cytoplasmic incompatibility (CI) (Yen & Barr 1971), parthenogenesis (Stouthamer et al. 1999), feminization (Rousset et al. 1992) and male killing (Hurst et al. 2000). CI results in the generation of unviable offspring when an uninfected female mates with a *Wolbachia*-infected male (McGraw et al. 2001). In contrast, *Wolbachia*-infected females can produce viable progeny when they mate with both infected and uninfected males resulting in a selective reproductive advantage over uninfected females (Hoffmann & Turelli 1997). This CI phenotype, shown in Figure, is induced by *Wolbachia* in mosquito species and allows the maternally transmitted *Wolbachia* to efficiently invade host populations without being infectious or moving horizontally between individuals (Hoffmann & Turelli 1997).

Although *Wolbachia* usually form non-virulent associations with their hosts (Sinkins et al. 1997), a virulent strain of *Wolbachia*, *w*MelPop, has been described that reduces the adult life span of its natural fruit fly host *Drosophila melanogaster* (Min & Benzer 1997). This *Wolbachia* infection appears to be quiescent in the developing immature stages of the fruit fly but enters into a stage of massive over replication once adult flies emerge. The life-shortening phenotype induced by the *w*MelPop strain was predicted to have potential use in the control of mosquito-borne diseases. As most pathogens need to develop within the mosquito vector for 8-21

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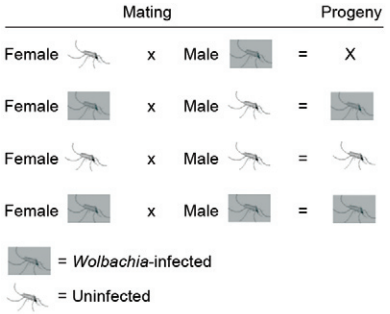

Wolbachia-induced cytoplasmic incompatibility in mosquitoes. The crossing patterns result in unhatched eggs when a *Wolbachia*-infected male mosquito mates with an uninfected female mosquito. *Wolbachia*-infected females produce infected progeny in all matings allowing the infection to rapidly spread through mosquito populations.

days post-infection, removal of older mosquitoes from the population, which are responsible for the majority of transmission, may result in substantial reductions in disease transmission (Sinkins & O'Neill 2000, Brownstein et al. 2003, Rasgon et al. 2003, Cook et al. 2008). However, the major vectors of human diseases such as *Anopheles* species (malaria) and *Aedes aegypti* (dengue) do not harbour natural *Wolbachia* infections. Therefore, the primary aim of using *Wolbachia* for human disease control is the stable transinfection of the bacteria into medically important mosquito vectors.

Wolbachia transinfection of Ae. aegypti mosquitoes - Successful transinfection of *Wolbachia* between distantly related insect species is dependent on the ability of the *Wolbachia* strain to adapt to new intracellular environments (Braig et al. 1994, Xi et al. 2005a). The *w*AlbB strain of *Wolbachia* was successfully established in *Ae. aegypti* using embryo cytoplasm transfer from closely related *Aedes albopictus* mosquitoes (Xi et al. 2005b). However, to facilitate the transfer of *Wolbachia* strains from more distantly related insect hosts, mosquito cell line adaptation appears to be critical for transinfection success. In order to facilitate the transfer of *w*MelPop from *D. melanogaster* fruit flies into *Ae. aegypti* mosquitoes, the bacteria was first transferred into mosquito cell lines to allow adaptation to the mosquito intracellular environment (McMeniman et al. 2008). After continuous serial passage in mosquito cell culture for over three years, the mosquito cell line-adapted *Wolbachia* strain, *w*MelPop-CLA, was stably introduced into *Ae. aegypti* using embryo microinjection (McMeniman et al. 2009). Two *Wolbachia*-infected lines were generated after a period of experimental selection in early generations and both lines remain highly infected four years after establishment.

The *w*MelPop-CLA strain results in approximately 50% reduction in the adult lifespan of *Ae. aegypti* mosquitoes (McMeniman et al. 2009, Yeap et al. 2011). This reduction in adult lifespan of female mosquitoes is predicted to result in a significant reduction in dengue transmission, if the capacity for life-shortening under laboratory conditions can be translated into field settings. Mosquito age is a critical factor for pathogen transmission (Dye 1992) as pathogens such as *Plasmodium* parasites undergoes an extrinsic incubation period (EIP) within the mosquito. The EIP is the time required from the ingestion of the pathogen until it is transmitted to the next vertebrate host. The speed of *Plasmodium* development within *Anopheles* mosquitoes depends on host, parasite and environmental factors such as temperature (Paaijmans et al. 2009). A typical incubation period of 10-14 days is relatively long compared to the longevity of adult mosquitoes (Charlwood et al. 1997, Killeen et al. 2000). In addition, there is typically at least two days from adult eclosion until adult female mosquitoes take their first bloodmeal. Adult mosquitoes also experience a high daily mortality rate resulting in only a small percentage of the total population actually surviving long enough to transmit malaria (Brownstein et al. 2003). Therefore, a reduction in the daily survival rates is likely to remove a large proportion of the mosquito population capable of transmitting malaria. Currently the most effective malaria vector control strategies, including indoor residual insecticide sprays and long-lasting insecticide-treated net, reduce the daily survival rates of *Anopheles* mosquitoes (Enayati & Hemingway 2010). Reducing the survival of mosquitoes leads to an approximately exponential decline in transmission intensity (Bellan 2010). Consequently, transinfection of life-shortening strains of *Wolbachia* into *Anopheles* would be predicted to significantly impact malaria transmission.

Wolbachia-induced pathogen interference - In *Drosophila*, the *w*MelPop and closely related *Wolbachia* strains have the capability of protecting against RNA virus infection by delaying the mortality of flies infected with a range of pathogenic viruses (Hedges et al. 2008, Teixeira et al. 2008). *Wolbachia* strains also provide significant pathogen protection in mosquitoes (Table). Infection of the *w*MelPop-CLA strain in *Ae. aegypti* also provides direct resistance against dengue infection (Moreira et al. 2009). When dengue serotype-2 (DENV-2) was introduced into *w*MelPop-CLA infected *Ae. aegypti* mosquitoes by either intrathoracic injection or oral feeding, an almost complete protection against viral infection was observed. The ability of *w*MelPop-CLA to interfere with viral replication also appears to occur with other arboviruses transmitted by *Ae. aegypti*, with similar virus interference effects for Chikungunya virus in oral feeding experiments of *w*MelPop-CLA infected *Ae. aegypti* (Moreira et al. 2009). There is also further evidence that the *w*MelPop-CLA strain provides protection against both filarial nematodes (Kambris et al. 2009) and avian malaria parasites (Moreira et al. 2009) suggesting some *Wolbachia* strains may inhibit a broad range of human pathogens.

As viral interference is not ubiquitous among *Wolbachia* strains (Moreira et al. 2009, Osborne et al. 2009) the mechanisms behind the ability of *Wolbachia* to provide resistance against pathogens are unknown. Although immune effector genes are upregulated in *w*MelPop-CLA infected *Ae. aegypti* mosquitoes, key components of the currently accepted signalling pathways for these effectors do not appear to be transcriptionally modulated by *Wolbachia* (Moreira et al. 2009, Kambris et al. 2010). Previous studies also revealed that some genes

TABLE

Wolbachia-induced pathogen protection in mosquitoes

Wolbachia strain	Mosquito species	Pathogen inhibited
*w*MelPop-CLA	*Anopheles gambiae*[a]	*Plasmodium berghei* Kambris et al. (2010) *Plasmodium falciparum* Hughes et al. (2011)
*w*MelPop-CLA	*Aedes aegypti*	*Plasmodium gallinaceum* Dengue Chikungunya Moreira et al. (2009) *Brugia pahangi* Kambris et al. (2009)
*w*AlbB	*Aedes aegypti*	Dengue Bian et al. (2010)
*w*Mel	*Aedes aegypti*	Dengue T Walker et al., unpublished observations
*w*Pip	*Culex quinquefasciatus*	West Nile virus Glaser and Meola (2010)

a: adult female mosquitoes were transiently infected through intrathoracic injection of *Wolbachia*. Transinfected (*w*MelPop-CLA, *w*Mel and *w*AlbB) and a natural strain (*w*Pip) of *Wolbachia* have been shown to directly inhibit pathogens in mosquitoes including two strains of *Plasmodium* parasites.

from the Toll, Imd and Jak-STAT pathways, implicated in the control of RNA virus infection in insects (Huszar & Imler 2008) are differentially regulated in *Ae. aegypti* mosquitoes infected with dengue (Xi et al. 2008). The ability of the *w*MelPop-CLA strain to provide dengue virus protection may also be dependent on competition for essential host cell components, as DENV-2 infection was only observed in cells of *w*MelPop-CLA infected mosquitoes that did not harbour *Wolbachia* (Moreira et al. 2009). Interestingly, cholesterol is a key fatty acid that is obtained from the insect host cell by both *Wolbachia* (Lin & Rikihisa 2003, Wu et al. 2004) and arboviruses (Lu et al. 1999, Mackenzie et al. 2007). In addition, *Plasmodium* is dependent on host lipids in the mosquito stage (Atella et al. 2009), suggesting cholesterol could be a critical host nutrient required by both *Wolbachia* and mosquito-borne pathogens.

The density and tissue distribution of *Wolbachia* infections in insect hosts may be an important determinant of their ability to interfere with pathogens. *Wolbachia* strains that provide protection in *Drosophila simulans* are closely related to the *w*MelPop strain in *D. melanogaster* and are found at comparatively high densities in flies (Osborne et al. 2009). The closely-related non-virulent *w*Mel strain also provides significant protection against DENV-2 in transinfected *Ae. aegypti* mosquitoes resulting in complete blockage of dengue transmission under experimental conditions (unpublished observations). Interestingly, strains of *Wolbachia* that naturally reside in mosquitoes show no or very limited capability for virus protection. *Ae. albopictus* mosquitoes are infected with non-virulent *w*AlbA and *w*AlbB strains of *Wolbachia* (Sinkins et al. 1995) yet are competent vectors of dengue virus (Kyle & Harris 2008). Similarly, *Armigeres subaltatus* mosquitoes are infected with a *Wolbachia* strain but no evidence is seen for interference with Japanese encephalitis virus (Tsai et al. 2006). *Aedes fluviatillis* mosquitoes are infected with a strain of *Wolbachia*, named *w*Flu, despite being competent laboratory vectors of *Plasmodium gallinaceum* (Moreira et al. 2009). Recently the native *w*Pip strain of *Wolbachia* in *Culex quinquefasciatus* was shown to have some protective effect against West Nile virus (Glaser & Meola 2010). However, this effect was much less pronounced when compared to the effects on dengue virus for transinfected fruit fly *Wolbachia* strains in *Ae. aegypti* (T Walker et al., unpublished observations). Overall it appears that the ability of *Wolbachia* to generate pathogen interference is likely to be restricted to *Wolbachia* strains that grow to high densities and have a wide tissue tropism in their insect host.

Wolbachia transinfection of Anopheles mosquitoes - The use of *Wolbachia* for malaria control will require a stable infection that is transmitted vertically to offspring, as occurs with fruit fly *Wolbachia* strains transinfected into *Ae. aegypti* mosquitoes (McMeniman et al. 2009, T Walker et al., unpublished observations). Natural *Wolbachia* infections had never previously been detected in any species of *Anopheles* (Curtis & Sinkins 1998, Kittayapong et al. 2000, Ricci et al. 2002, Tsai et al. 2004, Slotman et al. 2005). However, *Wolbachia*-positive individuals have recently been found in some species of *Anopheles* from the Amazon Region (RA Passos & WP Tadei, personal communication). Further studies on this finding can greatly open the potential use of this methodology for malaria control.

The challenging nature of mosquito microinjection has hampered the progress in transferring *Wolbachia* between mosquito species. The transfer of *Wolbachia*

into mosquito embryos is intrinsically more difficult than transfer into *Drosophila*, since mosquito embryos are less amenable to inoculation, especially *Anopheles* embryos. Factors such as the age at the time of injection and desiccation level are especially critical for both injectability and survival of *Anopheles* embryos. Using *Wolbachia*-infected mosquito cell lines, the generation of a stably infected line was not been possible despite microinjection of approximately 10,000 embryos of the malaria vectors *Anopheles stephensi* and *Anopheles farauti* (unpublished observations).

Wolbachia strains can be maintained in vitro in immunocompetent *Anopheles gambiae* cell lines (Rasgon et al. 2006, McMeniman et al. 2008), suggesting there is no intrinsic genetic mechanism preventing the infection of *Anopheles* cells with *Wolbachia*. In addition, when the *w*MelPop strain was injected into the hemolymph of *An. gambiae* adult females, the bacteria were able to survive and replicate in somatic tissues (Jin et al. 2009). However, the infection was not present in germline tissue (ovaries) so a stable transinfection could not be established using this methodology. The reason why *Wolbachia* does neither naturally infect *Anopheles* species nor form a stable infection is unknown. The ability to generate transient somatic infections through injection of adult mosquitoes (Jin et al. 2009, Kambris et al. 2010, Hughes et al. 2011) suggests the possibility that *Wolbachia* may be unable to colonize the germline tissue (ovaries). Successful transinfection requires infection of the germline prior to pole-cell formation in the pre-blastoderm stage of embryo development. Maternal transmission of *Wolbachia* to resulting progeny is dependent on establishing infections in the ovaries of adult females. Ultimately this barrier to germline infection must be overcome to establish stably infected lines that could be deployed for malaria vector control strategies. A recent additional study shows *Plasmodium falciparum* development in *An. gambiae* is surpressed by transient somatic infections of *w*MelPop-CLA (Hughes et al. 2011).

Wolbachia's effect on Plasmodium - Several studies present evidence that *Wolbachia* is likely to provide some protection against human malaria *Plasmodium* parasites if stable transinfection of *Anopheles* is achieved. The effect of the *w*MelPop-CLA strain on *P. gallinaceum* was tested as this species of malaria parasite is known to be able to infect *Ae. aegypti* mosquitoes in the laboratory. The *P. gallinaceum* oocyst load was reduced by 67-88% for *w*MelPop-CLA infected *Ae. aegypti* mosquitoes compared to *Wolbachia*-uninfected mosquitoes seven days after feeding on an infected chicken (Moreira et al. 2009). In *An. gambiae* females transiently infected with *w*MelPop using adult injection, mean *Plasmodium berghei* levels were reduced by 75-84% (Kambris et al. 2010). Although this combination of vector/parasite does not occur in nature, these results do highlight the ability of *Wolbachia* to significantly reduce the levels of malaria parasites in *Anopheles* mosquitoes.

Wolbachia invasion of wild Anopheles mosquito populations - An introduced *Wolbachia* infection in *Anopheles* mosquitoes would require induction of the CI phenotype and high rates of maternal transmission to successfully invade wild populations. The *w*MelPop and *w*Mel strains induce CI in transinfected *Ae. aegypti* mosquitoes (McMeniman et al. 2009, T Walker et al., unpublished observations), suggesting this phenotype would be induced in stably infected *Anopheles* lines. The maternal transmission rate of *w*MelPop-CLA in *Ae. aegypti* is estimated to be above 99% in laboratory lines (McMeniman et al. 2009), suggesting a high infection frequency could be achieved in transinfected *Anopheles* lines. The potential of *Wolbachia* to rapidly spread through insect populations under the action of CI was spatially described by Turelli and Hoffmann (1991) in wild *D. simulans* populations in California with the *w*Ri strain rapidly spreading at a rate of 100 km per year. This rapid spread of *Wolbachia* through wild *Drosophila* populations was described as a "Bartonian wave" (Turelli & Hoffmann 1991). The ability of any *Wolbachia* strain to successfully invade wild *Anopheles* mosquito populations will depend on an unstable threshold infection level. This unstable point depends on the negative selection imposed by fitness costs of *Wolbachia* infection and positive selection associated with CI induction (Hoffmann & Turelli 1997, Turelli 2010). If the threshold infection frequency is reached through introduction at an initial prevalence greater than the unstable equilibrium value, the *Wolbachia* infection is expected to spread to fixation over subsequent generations (Hoffmann & Turelli 1997, Turelli 2010).

Any fitness costs imposed by *Wolbachia* will raise the unstable point slowing the spread of infection through the "Bartonian wave" of invasion. The likely fitness costs associated with the stable introduction of the *w*MelPop-CLA strain into *Anopheles* will make this strain relatively weak at spreading into mosquito populations. Alternative *Wolbachia* strains such as *w*Mel that are predicted to impose less of a fitness cost to transinfected *Anopheles* mosquitoes are likely to be more successful at invasion of wild mosquito populations. The minimal fitness costs of *Wolbachia* infection are critical given the importance of mosquito fitness on the ability of released mosquitoes to compete with wild populations. A major advantage of a *Wolbachia*-based biocontrol approach for malaria is that CI acts as a self-spreading mechanism for *Wolbachia* to rapidly invade populations from the release of relatively small numbers of individuals. The predicted direct inhibition of human *Plasmodium* parasites by *Wolbachia* may also augment CI as a broad based mechanism for population invasion to provide a positive fitness benefit to *Anopheles* mosquitoes carrying *Wolbachia*. This may overlay the traditional "Bartonian" view of CI based invasion dynamics and provide an additional driving force for *Wolbachia* depending on the extent of fitness advantages conferred to field populations.

Ultimately the use of *Wolbachia* for malaria control will require stably infected lines of major malaria vectors such as *An. gambiae s.l.* (Africa), *An. stephensi* (India) and *An. darlingi* (Central and South America) and a comprehensive assessment of the protective effect against human malaria parasites such as *P. falciparum* and *P. vivax*. The applied use of *Wolbachia* for malaria control would also require significant characterization

of *Wolbachia*'s phenotypic effects in diverse genetic background of these *Anopheles* vector species. In reality, widespread control of malaria using *Wolbachia*-based methods is not likely achievable. For example, the difficulties of colonizing *An. darlingi* (and therefore transinfecting this species with *Wolbachia*) would prevent the applied use of *Wolbachia* for control of malaria in parts of the Amazonian Region. In that case, transinfection of colonisable species such as *Anopheles aquasalis* (Da Silva et al. 2006) would provide applicability in areas where this species has vectorial importance.

Lastly, one has to be aware that the complexity of malaria vector populations (Lanzaro et al. 1998, Donnelly et al. 2002) would be a major complicating factor in the applied use of *Wolbachia* for malaria control. However, this novel approach may provide an effective mechanism of malaria control in some malaria endemic areas in which a single, vector species is present.

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