8. *Wolbachia* in *Aedes* mosquitoes: towards biological control of vector-borne diseases

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Abstract

Dengue causes an enormous impact on global public health with 50 million cases every year. Vector control is predominantly focused on the application of insecticides against adult mosquitoes. However, current methods of vector control are often not sustainable for long periods because of the emergence of insecticide resistance. In this context, the discovery and application of alternative methods are extremely important. A new approach for biological control of diseases transmitted by mosquitoes has recently been proposed that uses an endosymbiotic bacterium (*Wolbachia pipientis*) in order to interfere with the transmission of pathogens. The advantage of using this bacterium is that infected females have a reproductive advantage due to a cytoplasmic incompatibility (CI), which leads to an increase in numbers of infected individuals in the wild. When *Aedes aegypti* mosquitoes (that naturally lack this bacterium) were transinfected with *Wolbachia* it was discovered that the presence of bacteria inhibits the replication of pathogens such as dengue and chikungunya viruses as well as filarial and avian malaria parasites. More recently *Ae. aegypti* mosquitoes harbouring a *Drosophila-Wolbachia* strain have been released in Australia and were able to quickly spread in the wild. In this chapter we review the possibility of applying this endosymbiotic bacteria as potential biological control of human diseases transmitted by mosquito vectors.

Keywords: *Aedes*, biological control, dengue, *Wolbachia*

Introduction

Vector-borne diseases such as malaria, leishmaniasis and dengue heavily impact on human mortality and morbidity throughout the world. With the increase of human movement (Adams and Kapan 2009) and the effects of global warming (Barclay 2008, Pachauri and Reisinger 2007) the expansion and resurgence of pathogens (Gould and Solomon 2008) such as dengue (DENV) and chikungunya (CHIKV) are becoming an increasing threat (Ng and Ojcius 2009, Staples *et al.* 2009). Dengue fever is being recently named as the most important disease affecting communities in tropical and sub-tropical regions around the world with 50 million cases annually and causing thousands of deaths (WHO 2009). A new approach to dengue control was proposed that targets mosquito longevity rather than their abundance, by introducing a strain of the bacterium *Wolbachia pipientis* (Hertig and Wolbach 1924), which causes a reduction in survival of *Aedes aegypti* L. mosquitoes (Brownstein *et al.* 2003, Cook *et al.* 2008, Rasgon *et al.* 2003, Sinkins and O'Neill 2000). Since the extrinsic incubation period of viruses and parasites within the mosquito vector is long (about 15 days) compared with the longevity of the insect (about 30 days in the field), the *Wolbachia* infection that may invade the mosquito population and causes a reduction in longevity of *Ae. aegypti*, is expected to reduce the transmission of pathogens without eliminating the mosquito population (Brownstein *et al.* 2003, Rasgon *et al.* 2003, Sinkins and O'Neill 2000). The *Wolbachia* wMelPop-CLA strain originating from *Drosophila melanogaster* Meigen was successcully introduced into *Ae. aegypti* (McMeniman *et al.* 2009). Mosquitoes containing this strain of *Wolbachia* died significantly sooner compared to their counterparts that lacked bacteria. Besides the effect on the insect longevity, and with great surprise was the discovery that the presence of bacteria in mosquitoes increases the resistance towards pathogens (Bian *et al.* 2010, Kambris *et al.* 2009, Moreira *et al.* 2009, Walker *et al.* 2011) as also shown in *Drosophila* flies for fly-specific viruses (Hedges *et al.* 2008, Teixeira *et al.* 2008). In this chapter we discuss the potential use of *Wolbachia* in biological control of diseases transmitted by mosquito vectors.

Phenotypic effects of *Wolbachia* in mosquitoes

Wolbachia are Gram-negative, obligatory intracellular bacteria, which manipulate host reproduction to ensure vertical transmission (from mother to offspring) (Sinkins *et al.* 1997). In the last decades *Wolbachia* was widely found infecting different species of invertebrates, with reports on arthropods such as insects (Jeyaprakash and Hoy 2000, Stouthamer *et al.* 1999, Werren *et al.* 1995), arachnids (Breeuwer and Jacobs 1996), crustaceans (Cordaux *et al.* 2001, Gotoh *et al.* 2003) and isopods, although they are also found in nematodes (Bandi *et al.* 1998, 2001). The first report of *Wolbachia* was in the reproductive tissues of *Culex pipiens* L. (Hertig and Wolbach 1924), being named *Wolbachia pipientis* (Hertig 1936). Recent statistical analysis confirmed the wide distribution of this bacterium among invertebrates, with an estimate of positivity in up to 65% of all insect species (Hilgenboecker *et al.* 2008), confirming previous findings of infection of 20 to 70% of insect species (Jeyaprakash and Hoy 2000).

In order to guarantee vertical transmission, the bacteria manipulate their hosts in various ways such as feminisation, male killing, parthenogenesis as well as through the mechanism of cytoplasmic incompatibility (Figure 1). As the latter is the most common phenomenon in mosquitoes this effect will be briefly explained below.

Cytoplasmic incompatibility

Cytoplasmic incompatibility (CI) is the most common effect caused by *Wolbachia* on arthropod reproduction. The phenotype results in the production of aberrant progeny originating from strains that harbour insects and various cytoplasmic factors that will affect the proper assembly of chromosomes in sperm soon after fertilisation. Typically, the paternal chromosomes are eliminated, leading to the formation of haploid embryos. In eggs of incompatible crosses, only the female pronucleus forms individual chromosomes and proceeds to the first division. The paternal

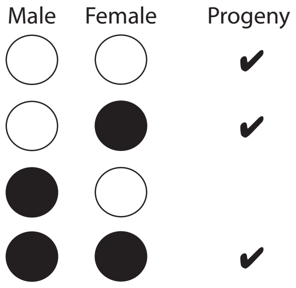

Figure 1. Unilateral mechanism of cytoplasmic incompatibility (CI). Crosses between infected males with uninfected females produce no viable offspring due to CI. Crosses involving Wolbachia*-infected females (in black) have higher chance of producing viable offspring, increasing the number of infected individuals in the population.*

pronucleus, which appears as a mass of chromatin tends to fragment during the first mitotic division. The effect of CI is usually unidirectional: incompatible crossing occurs between infected males and uninfected females, whereas reciprocal crosses between males free of bacteria and infected females produce normal offspring (Figure 1). However, there are reports of bidirectional incompatibility when species of insects (usually mosquitoes and *Drosophila*) hosts more than one strain of bacteria (Stouthamer *et al.* 1999, Yen and Barr 1971).

Wolbachia in *Aedes* mosquitoes

Early studies involved observations of *Rickettsia*-like organisms within mosquito ovaries and eggs. Following the work of Hertig in *Culex* (Hertig 1936), the first report of *Wolbachia* in mosquitoes came with the work by Yen (1975) on members of the *Aedes scutellaris* group (such as *Aedes cooki* Belkin*, Aedes polynesiensis* Marks*, Aedes albopictus* Skuse*, Aedes riversi* Bohart & Ingram). The ultrastructure of *Wolbachia* was then thoroughly studied in this same group by Wright and colleagues, who identified round shape structures within mosquito ovaries. They claimed that the nurse cells of the scutellaris group (unlike those in *Cx. pipiens*) are rarely infected with *Wolbachia* (Wright and Barr 1980).

The Asian tiger mosquito *Ae. albopictus* is native to Asia and the South Pacific and is an important vector of dengue and chikungunya viruses in some places of Southeast Asia (Kumari *et al.* 2011, Ratsitorahina *et al.* 2008, Rudnick and Chan 1965, Tsetsarkin *et al.* 2011). *Wolbachia* infections were first found in the ovaries of this mosquito (Dobson *et al.* 2001, 2004, Sinkins *et al.* 1995a, Wright and Wang 1980). Today it is well known that nearly all populations of *Ae. albopictus* harbour two different *Wolbachia* strains named *w*AlbA (A group) and *w*AlbB (B group) (Kittayapong *et al.* 2000, Ruang-Areerate *et al.* 2003, Sinkins *et al.* 1995a). Studies on the cytoplasmic incompatibility between both strains, towards the potential use of this feature as a mean of population suppression, have been performed by several groups (Dobson *et al.* 2001, 2004, Sinkins *et al.* 1995a, Tortosa *et al.* 2010).

Interestingly *Wolbachia* has never been found in wild *Ae. aegypti* (Kittayapong *et al.* 2000) nor in any anopheline mosquitoes (vectors of human malaria parasites) (Kittayapong *et al.* 2000, Rasgon and Scott 2004b, Ricci *et al.* 2002). The reason for this is unknown.

Transinfection of *Wolbachia* into *Aedes aegypti*

The success of *Wolbachia* transinfection between different insect taxa is dependent on the ability of this bacterium to adapt to new intracellular environments (Braig *et al.* 1994, Xi *et al.* 2005a). An example of successful transinfection in species of same genus involved the wAlbB *Wolbachia* strain from *Ae. albopictus* which was successfully established into *Ae. aegypti* using cytoplasmic transfer of embryos (Xi *et al.* 2005b). *Wolbachia*-infected mosquitoes exhibit cytoplasmic incompatibility and cage experiments have demonstrated that infected mosquitoes were able to reach fixation within seven generations (Xi *et al.* 2005b). Also, both *Wolbachia* strains from *Ae. albopictus* (*w*AlbA and *w*AlbB) were injected into *Ae. aegypti* adults and infected strains were able to persist over several generations (Ruang-Areerate and Kittayapong 2006).

However, the transfer of *Wolbachia* strains from other groups of insects seems to follow a prerequisite of cellular adaptation to the mosquito, which can be regarded as critical for successful transinfection. Thus, in order to facilitate the transfer of *Wolbachia* from *D. melanogaster* cells to *Ae. aegypti*, the *w*MelPop strain was first transferred to a mosquito cell line to allow adaptation to that intracellular environment (McMeniman *et al.* 2008). After continuous serial passage in mosquito cell cultures for more than three years, the mosquito cell line became adapted to the strain of *Wolbachia,* named *w*MelPop-CLA (CLA-cell line adapted), which was later stably introduced into *Ae. aegypti* by microinjection of embryos (McMeniman *et al.* 2009). Two positive-*Wolbachia* strains were generated after a trial period of selection in early generations and both strains remained highly infected since then. Laboratory experiments using *Ae. aegypti* infected with wMelPop-CLA showed that the bacteria decreased adult longevity with approximately 50% (McMeniman *et al.* 2009, Yeap *et al.* 2011). This reduction in life expectancy of female *Ae. aegypti* may result in a significant decrease in the transmission of dengue virus by mosquitoes, if this ability to shorten the life in the laboratory ought to be reproduced under field conditions.

It is well known that the mosquito age is a critical factor for the transmission of pathogens (Dye 1992) as viruses and parasites go through an extrinsic incubation period (EIP) within the mosquito. The EIP is the time from ingestion of the pathogen until it is transmitted to the next vertebrate host and is a key component to calculate the vectorial capacity of diseases (Meyer 1989).

Influence of *Wolbachia* towards host-pathogen interactions

The interaction of *Wolbachia* with their hosts has the possibility of directly affecting their fitness (positively or negatively) or being regarded as silent or neutral. Therefore, the relation between the bacterium and the host can be regarded as mutualistic or parasitic (Brownlie *et al.* 2009, Werren *et al.* 2008).

In *Armadillidium vulgare* Latreille it has been shown that a particular strain of *Wolbachia* (*w*VulC) lowers haemocyte densities, increases septicaemia in their haemolymph and reduces their lifespan compared to individuals harbouring another bacterium strain or aposymbiotic (*Wolbachia*-free) ones (Braquart-Varnier *et al.* 2008). This phenomenon directly points to the fact that this microorganism can affect its host immunity. In another study, *Drosophila* infected with *Wolbachia* exhibited lower levels of encapsulation of parasitic wasp eggs than cured ones (Fytrou *et al.* 2006).

Recent studies in *Drosophila* have shown that infection with *Wolbachia* can protect flies from infection with RNA viruses (Hedges *et al.* 2008, Teixeira *et al.* 2008). The *Wolbachia* wMelPop and wMelCS strains that infect *D. melanogaster* induced reduction in mortality when the flies were infected with various pathogenic viruses including the *Drosophila* C virus, Flock House virus (FHV) and locusts paralysis virus (Cricket paralysis virus).

As the strain of *Wolbachia* wMelPop promoted protection against RNA viruses in *Drosophila*, it was important to test the effect of wMelPop-CLA on the vectorial competence of *Ae. aegypti* transinfected lines (McMeniman *et al.* 2009). For that, mosquitoes infected and uninfected with *Wolbachia* were exposed to dengue and Chikungunya viruses. The same mosquitoes were also tested with the avian malaria parasite, *Plasmodium gallinaceum*. The results showed that the presence of *Wolbachia* caused a drastic reduction in the presence or the development of these three unrelated pathogens, which opens new possibilities for controlling mosquito-borne diseases (Moreira *et al.* 2009). More recently it was shown that the bacterium (wMelPop-CLA) also provides protection against nematodes that cause lymphatic filariasis (Kambris *et al.* 2009) and blocked *Plasmodium falciparum* in somatically infected *Anopheles gambiae* Giles mosquitoes (Hughes *et al.* 2011), suggesting that some strains of *Wolbachia* may inhibit a wide range of human pathogens.

As viral interference is not ubiquitous among the strains of *Wolbachia* (Moreira *et al.* 2009, Osborne *et al.* 2009) the mechanisms behind the ability of bacteria to gain resistance against pathogens are unknown. Although some effector genes (such as defensin, cecropin) were up-regulated in *Ae. aegypti* mosquitoes infected with *w*MelPop-CLA, key components of signalling pathways (Toll, IMD and Jak-STAT) do not appear to be transcriptionally modulated by *Wolbachia* (Kambris *et al.* 2010, Moreira *et al.* 2009). Previous studies have also revealed that some genes of the IMD and Jak-STAT pathways, involved in the control of infection by RNA viruses in insects (Huszar and Imler 2008), are differentially regulated in *Ae. aegypti* infected with dengue (Xi *et al.* 2008).

The ability of the CLA-wMelPop strain to provide protection against dengue virus may also be dependent on competition for essential components of host cells, as observed in infection with DENV-2 in cells infected with mosquito-CLA wMelPop (Moreira *et al.* 2009). Moreover, in *D. melanogaster* there is evidence that strains of *Wolbachia* get much of their energy through the metabolism of amino acids (Wu *et al.* 2004), including threonine – an amino acid required in the activation of expression of vitellogenin (Vg) in *Ae. aegypti* (Attardo *et al.* 2006). Recently McMeniman and colleagues hypothesized the existence of a competition between wMelPop *Wolbachia* and *Ae. aegypti* to obtain threonine required for expression of Vg and subsequently inhibition of egg development (McMeniman *et al.* 2011). Alternatively, it is known that insects also need to get cholesterol and other fatty acids in the diet (Blitzer *et al.* 2005) and, as the *Wolbachia* and other bacteria do not synthesise cholesterol, they might need to get it from the insect host (Lin and Rikihisa 2003, Wu *et al.* 2004). Cholesterol is known to be a key fatty acid necessary for successful replication of flaviviruses and that must be obtained from the host cell (Lu *et al.* 1999, Mackenzie *et al.* 2007). Likewise *Plasmodium* also depend on the mosquito lipids (Atella *et al.* 2009), suggesting that cholesterol may be a critical nutrient required by both the pathogen and the *Wolbachia* within the mosquito.

The distribution of *Wolbachia* in different tissues of the mosquito, as well as the density of the same insect host cells may be an important determinant in the ability of bacteria to interfere with the pathogens. *Wolbachia* strains that provide protection in *Drosophila simulans* Sturtevant, are closely related to wMelPop from *D. melanogaster* and are also found in high densities in flies (Osborne *et al.* 2009). But recently, the non-virulent strain *w*Mel (also from *Drosophila*), although present in much lower densities than *w*MelPop, promotes significant protection against DENV-2 in transinfected *Ae. aegypti* mosquitoes, resulting in a total blockage of dengue transmission under experimental conditions (Walker *et al.* 2011). Perhaps, a combination of bacterial tissue tropism, host immunity upregulation and competition for host cell resources is what is needed for pathogen blockage.

Contrarily to observations in *Drosophila*, the strains of *Wolbachia* (Hedges *et al.* 2008, Teixeira *et al.* 2008) which naturally reside in mosquitoes, have very limited ability to protect against viruses. *Ae. albopictus* infected with the non-virulent strains of *Wolbachia* (wAlbA and wAlbB) (Sinkins *et al.* 1995b) are still vectors of dengue virus (Kyle and Harris 2008). Likewise, *Armigeres subalbatus* Coquillett mosquitoes infected with another strain of *Wolbachia* showed no evidence of interference with the Japanese encephalitis virus (Tsai *et al.* 2006). Recently, the strain of *Wolbachia w*Pip native of *Culex quinquefasciatus* Say, was shown to have some protective effect against West Nile virus (Glaser and Meola 2010). However, this effect was much less pronounced when compared with the effects on dengue virus in transinfected *Ae. aegypti* (Moreira *et al.* 2009, Walker *et al.* 2011).

Practical application

The practical approach of using *Wolbachia* to control the spread of mosquito-borne diseases has been around for a long time (Curtis and Sinkins 1998, Rasgon and Scott 2004a, Sinkins *et al.* 1997). The mechanism of CI has been experimentally used in the wild in order to eradicate a population of *Cx. quinquefasciatus* (formerly: *Culex pipiens fatigans*) in Burma. Although adult numbers decreased to very low levels, the authors were not able to completely eliminate the local vector population (Laven 1967). More than a decade ago it has been proposed to use *Wolbachia* as a tool for spreading gene(s) of interest in field populations. The initial idea was to transform this bacterium by placing, for example, a refractoriness gene and, with the action of cytoplasmic incompatibility (CI) the bacteria would invade the wild population carrying the gene of interest (Curtis and Sinkins 1998). However since then, several laboratories have been trying to transform the bacteria with no success and the inability of this microorganism to live in a cell free medium appears to be the main impairment.

The CI mechanism has been proposed as an alternative to the Sterile Insect Technique (SIT) where males harbouring a different *Wolbachia* strain from the one present in one particular area could be released and suppress local mosquito population by effectively sterilizing their female counterparts (Calvitti *et al.* 2010). *Ae. polynesiensis* is the main vector of human filariasis in the South Pacific. The use of incompatible *Wolbachia*-infected mosquito strains is been sought as infected males can be released and then decrease natural mosquito populations through the CI mechanism (Chambers *et al.* 2011).

The discovery that certain strains of *Wolbachia* may interact with pathogens by blocking their development in mosquitoes shifted the focus for the use of this bacterium and the previous life-shortening approach has been put on hold in favour of other, more effective, strategies. More recently, the use of *Wolbachia*-transinfected mosquito strains shows the potential of causing significant effects in disease control programmes around the world.

After a long and broad risk analysis step performed by two Australian research and regulatory agencies (CSIRO and APVMA) (Murphy *et al.* 2010) the release of *Wolbachia*-infected mosquitoes in the wild has been approved (DeBarro *et al.* 2011). In the beginning of January 2011 two localities that previously had undergone through a vast programme of community engagement, received the first *Wolbachia*-positive *Ae. aegypti* mosquitoes in nature (http://www.eliminatedengue.org). This process was followed by 9-10 weeks of adult mosquitoes releases and even with the occurrence of a cyclone they were able to invade natural populations, reaching up to 100% after the introduction has been halted (Hoffmann *et al.* 2011) (Figure 2). Soon the same approach will be used in other countries as Vietnam, Thailand, Indonesia and Brazil. If the *Wolbachia*-positive mosquitoes released in the field have the same ability of blocking the dengue virus the way it has been shown in laboratory conditions it will bring a big impact on disease control in the world. Further studies using the available *Wolbachia-Aedes* strains towards other dengue serotypes and other arboviroses such as yellow fever are most welcomed to expand the potential use of this strategy towards other diseases (Van den Hurk *et al.* 2012).

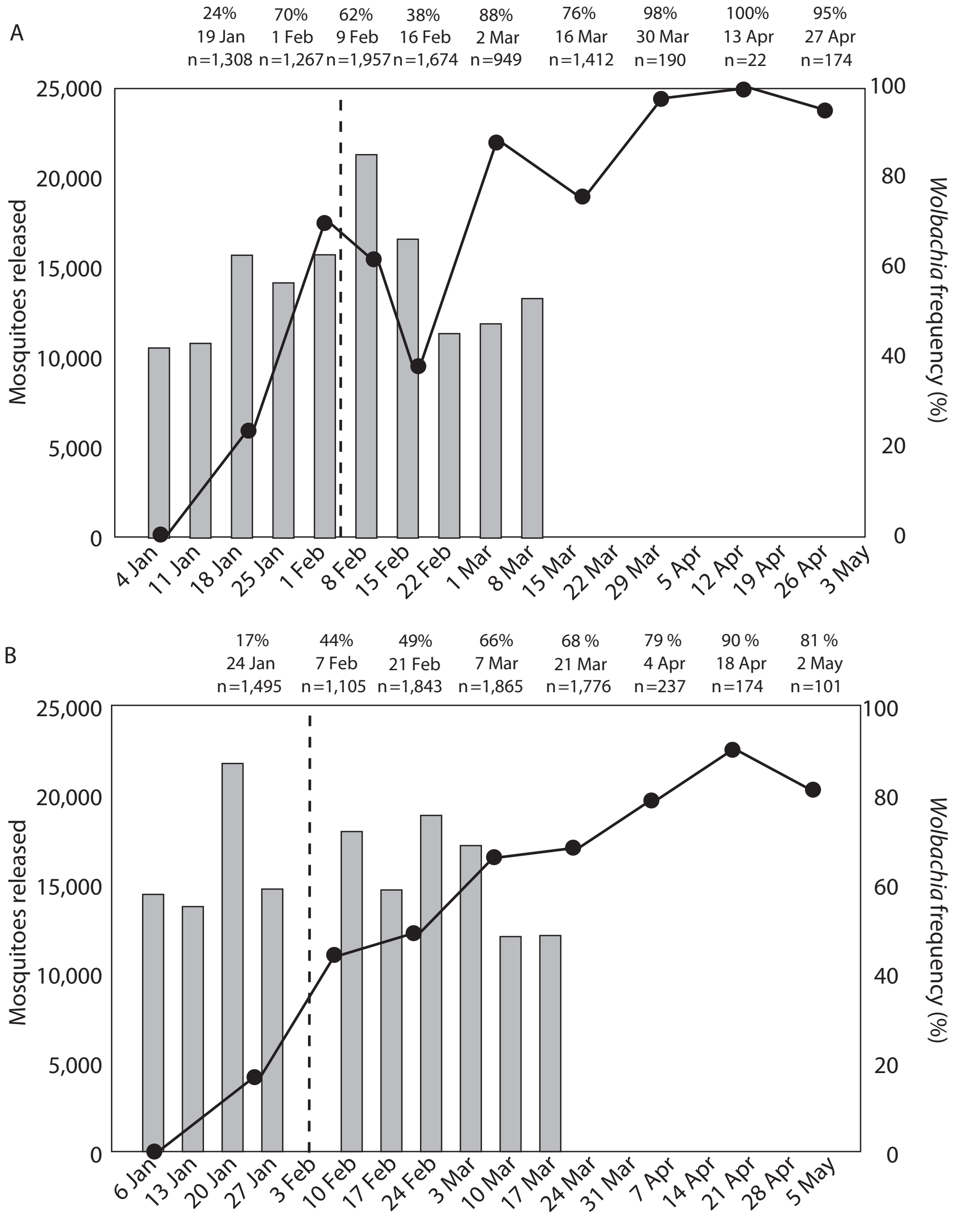

Figure 2. Number of mosquitoes released, timing of releases, and changes in infection frequencies over time. Data based on monitoring with ovitraps at (A) Yorkeys Knob and (B) Gordonvale. Ten releases were carried out at each site. Lower numbers were collected late in the season because of a reduction in trapping intensity and the advent of the dry season. Tropical Cyclone Yasi landed on 3 February (dotted line) and disrupted Wolbachia *monitoring collections at Yorkeys Knob. A planned release at Gordonvale on 3 February was cancelled (Hoffmann* et al. *2011).*

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