Short note:
An update on the utility of *Wolbachia* for controlling insect vectors and disease transmission

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**Introduction**

*Wolbachia pipientis* is a maternally inherited intracellular bacterium that is widespread among insect taxa, but is also found in spiders, terrestrial crustaceans, and filarial nematodes (Werren *et al.*, 2008). In arthropods, *Wolbachia* is responsible for inducing a number of reproductive modifications that enable its spread and maintenance in natural populations. The most studied reproductive modification is cytoplasmic incompatibility (CI) (Werren *et al.*, 2008), which has received considerable attention as a mechanism to control insect vectors of disease. *Wolbachia*-based strategies have primarily taken the form of population replacement strategies and the incompatible insect technique (IIT) that harness CI, reviewed in (Bourtzis, 2008; Brelsfoard and Dobson, 2009; Saridaki and Bourtzis, 2010). Recently there has been a substantial increase in *Wolbachia* research related to the interaction of *Wolbachia* and its host and impacts on parasite transmission. Findings have prompted researchers to propose strategies that utilize *Wolbachia* infections that limit parasite proliferation in the insect host to aid in the control of disease transmission. Also, an important step has been taken for *Wolbachia*-based control strategies, the release of *Wolbachia*-infected mosquitoes in a natural population with the ultimate goal of aiding in the control of mosquito-transmitted disease. This note discusses recent advances in *Wolbachia* research focused on insect control, and is intended to supplement a previous review (Brelsfoard and Dobson, 2009).

**The *Wolbachia*-host interaction: potential for insect vectored disease control**

**Virus resistance.** Recent developments using *Drosophila* as a model have suggested that *Wolbachia* confers resistance to virus infections in their hosts. A strain of the fruit fly *Drosophila melanogaster* that is naturally infected with *w*Mel was shown to reduce virus proliferation in *Drosophila* C (DCV), cricket paralysis (CrPV), Nora, and Flock House viruses (FHV) (Teixeira *et al.*, 2008) (Table 1). Similarly, a comparison of natural infections and introduced infections in the fruit fly *Drosophila simulans* challenged with DCV and FHV viruses suggested some infections induce an anti-viral response while others do not (Osborne *et al.*, 2009) (Table 1). The *Wolbachia* infections in this study that induced an anti-viral response were found in higher densities when compared to other *Wolbachia* infections found in *D. simulans*. The *Wolbachia* infections that induced an antiviral response were also most closely related to the *w*Mel infection type, suggesting *w*Mel or infections closely related to *w*Mel might induce antiviral responses in their host. These observations suggest that the anti-viral response may not be ubiquitous in *Wolbachia*-infected insects.

The observations in *Drosophila* have led researchers to investigate similar effects in medically important mosquito vectors. Bian *et al.* (2010) demonstrated that *Aedes aegypti* artificially infected with *w*AlbB have reduced Dengue virus proliferation compared to naturally uninfected strains. Feeding assays using
Table 1. Summary of recent publications describing *Wolbachia* effects on pathogen inhibition.

<table>
<thead>
<tr>
<th>Host</th>
<th>Wolbachia type</th>
<th>Pathogen</th>
<th><em>Wolbachia</em> effect on pathogen inhibition</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Aedes aegypti</em></td>
<td><em>w</em>AlbB</td>
<td>DV</td>
<td>Reduced virus proliferation</td>
<td>Bian <em>et al</em>., 2010</td>
</tr>
<tr>
<td></td>
<td><em>w</em>MelPop</td>
<td>DV, CHV, <em>Plasmodium gallinaceum</em></td>
<td>Reduced virus proliferation, decrease oocyst accumulation</td>
<td>Moreira <em>et al</em>., 2009b</td>
</tr>
<tr>
<td></td>
<td><em>w</em>MelPop</td>
<td>Brugia pahangi</td>
<td>Inhibition of filarial worm development</td>
<td>Kambris <em>et al</em>., 2009</td>
</tr>
<tr>
<td></td>
<td><em>w</em>Mel</td>
<td>DV</td>
<td>Blockage of viral proliferation</td>
<td>Walker <em>et al</em>., 2011</td>
</tr>
<tr>
<td><em>Drosophila melanogaster</em></td>
<td><em>w</em>Mel</td>
<td>DCV, GrPV, Nova, FHV</td>
<td>Reduced virus proliferation</td>
<td>Teixiera, 2008</td>
</tr>
<tr>
<td></td>
<td><em>w</em>Mel</td>
<td>WNV, CHV, LaV</td>
<td>Reduced virus proliferation</td>
<td>Glaser and Meola, 2010</td>
</tr>
<tr>
<td><em>Culex quinquefasciatus</em></td>
<td><em>w</em>Pip</td>
<td>WNV</td>
<td>Reduced virus proliferation</td>
<td>Glaser and Meola, 2010</td>
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<td><em>Drosophila simulans</em></td>
<td><em>w</em>Ri</td>
<td>FHV, DCV</td>
<td>Reduced virus proliferation</td>
<td>Osborne <em>et al</em>., 2009</td>
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<td></td>
<td><em>w</em>Au</td>
<td>FHV, DCV</td>
<td>Reduced virus proliferation</td>
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<td>FHV, DCV</td>
<td>No effect</td>
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<td><em>w</em>No</td>
<td>FHV, DCV</td>
<td>No effect</td>
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<td></td>
<td><em>w</em>Mel</td>
<td>FHV, DCV</td>
<td>Reduced virus proliferation</td>
<td></td>
</tr>
<tr>
<td><em>Anopheles gambiae</em></td>
<td><em>w</em>MelPop</td>
<td><em>Plasmodium berghei, Brugia pahangi</em></td>
<td>Decreased oocyst accumulation, reduced filarial worm development</td>
<td>Kambris <em>et al</em>., 2010</td>
</tr>
<tr>
<td><em>Aedes albopictus</em></td>
<td><em>w</em>AlbA and <em>w</em>AlbB</td>
<td>CHV</td>
<td>No effect</td>
<td>Mousson <em>et al</em>., 2010</td>
</tr>
</tbody>
</table>

Abbreviations: DCV - *Drosophila* C virus; FHV - Flock House Virus; GrPV - Cricket Paralysis Virus; WNV - West Nile Virus; DV - Dengue virus; CHV - Chikungunya Virus; LaV - La Crosse Virus.

blood infected with Dengue viruses showed reduced viral replication in *Wolbachia*-infected mosquitoes midgut and thorax, with few mosquitoes developing disseminated viral infections (Table 1). *Wolbachia* infections in *Culex quinquefasciatus* have also been observed to increase resistance to West Nile Virus (WNV) transmission (Glaser and Meola, 2010). *Wolbachia*-infected mosquitoes were observed to produce lower viral titers and have 2 to 3-fold lower rates of transmission compared to *Wolbachia*-uninfected mosquitoes. In the same study, *Wolbachia* infected *Drosophila melanogaster* was observed to have a 100,000 fold lower virus titer compared to *Wolbachia*-uninfected flies. However, *Wolbachia* infection in *D. melanogaster* had less of an effect on the susceptibility of the flies to Chikungunya and La Crosse viruses (Table 1). The naturally superinfected *w*AlbA and *w*AlbB *Aedes albopictus* showed an increase in Chikungunya virus replication compared to *Wolbachia*-uninfected controls. The increase in virus replication was also associated with a decrease in *Wolbachia* density (Mousson *et al*., 2010) (Table 1), suggesting an interactive effect of *Wolbachia* and Chikungunya virus. Further work is needed to determine if it is indeed *Wolbachia* density that affects virus replication or vice versa. Additional studies should investigate the vector competence of Chikungunya virus in the recently developed artificially *Wolbachia*-infected strain of *A. albopictus* (Calvitti *et al*., 2010).

The *w*MelPop infection has been shown to reduce the lifespan of its mosquito, whether the life shortening effect is below that of the extrinsic incubation time period of the Dengue virus in wild-type flies remains to be seen (McMeniman *et al*., 2009). While the reduction of adult longevity is novel and may offer a new tool to control vector borne disease, *w*MelPop infected *A. aegypti* has also been shown to be resistant to Dengue virus transmission and Chikungunya virus (Moreira *et al*., 2009b). No mosquitoes challenged with Dengue or the Chikungunya virus developed disseminated viral infections. Walker *et al*., (2011) introduced the *w*Mel infection into *A. aegypti* and demonstrated that the *Wolbachia*-infected mosquitoes are resistant to
Dengue virus transmission, display high rates of CI, and do not negatively affect host fitness, unlike that of wMel infections (Table 1). Using laboratory populations Walker and colleagues also demonstrated that the wMel infection could spread into a population at a rate consistent with theoretical model predictions.

**Resistance to other pathogens** wMelpop-infected *A. aegypti* individuals have also been shown to have half the number of developing *Plasmodium gallinaceum* oocysts (the life stage of the malaria parasite that is transmittable by an insect vector) compared to uninfected controls (Moreira et al., 2009) (Table 1). Kambris et al. (2009) demonstrated a wMelPop infection in *A. aegypti* challenged with the filarial worm *Brugia pahangi* reduced the infective state of the worm (L3) compared to *Wolbachia*-uninfected strains (Table 1). These results suggest an effect of *Wolbachia* on filarial worm development in the mosquito. Furthermore, as part of the same study Kambris et al. (2009) demonstrated that wMelPop infected mosquitoes exhibited a higher survivorship after intrathoracic inoculation with the bacterium *Erwinia carotovora* when compared to uninfected controls, suggesting that the *Wolbachia* infection is responsible for a protective effect in *A. aegypti*. Transient somatic wMelPop infections have also been established in the primary vector of malaria in Africa, *Anopheles gambiae*. Significant reductions of *Plasmodium berghei* oocysts were observed in wMelPop infected mosquitoes (Kambris et al., 2010) (Table 1). These experiments were performed using somatic infections in *A. gambiae*, but suggest the effects may be transient and will be the same if a maternally inherited *Wolbachia* infection can be generated. Furthermore, the previously described experiments were performed with species of *Plasmodium* that is not a human parasite, suggesting that the experiments will ultimately need to be repeated with a human-infecting *Plasmodium* species.

**Immune response induces pathogen inhibition**

Results from the aforementioned experiments point to a direct effect of *Wolbachia* infections on mosquito physiology, specifically induction of an immune response. Xi et al. (2008) showed an up regulation of the immune genes, Toll and Relish, in a wRi-infected *Drosophila* S2 cell line compared to *Wolbachia*-uninfected cells. Bian et al. (2010) recently demonstrated that there was an increased immune response in *Wolbachia*-infected *A. aegypti*. Several immune genes typically involved in the response to gram-negative bacterial infections (i.e. Defensin, Cercropin, Dipterican, GNBPB1, SPZ1A, Cactus, Rel1, and Rel2) were upregulated in response to the introduced *Wolbachia* infections. Results suggest that the immune response in the insect, potentially induced by the *Wolbachia* infection, may be responsible for the increased resistance to Dengue transmission. Similarly, Kambris et al. (2009) demonstrated the upregulation of several immune genes in comparisons between *Wolbachia*-infected and uninfected *A. aegypti*, suggesting that *Wolbachia* infection inhibits the development of filarial nematodes. The upregulation of six immune genes (LRIM1, TEP1, CEC1, DEF1, CTL4, and CLIPB3) was observed in wMelPop somatically infected *A. gambiae* and MOS55 *A. gambiae* cell lines (Kambris et al., 2010). As further evidence for the upregulation of the immune system, a TEP1 knockdown was performed by injecting dsRNA with wMelPop. A significantly higher number of oocysts were observed in knockdown treatments compared to controls, suggesting *Wolbachia* infection induces an immune response that affects oocyst proliferation. In contrast to the wMelPop *A. gambiae* infected cell lines, the comparison of wRi and wAlbB infected and uninfected *A. gambiae* cell lines (Sua5B) showed that *Wolbachia* significantly down regulates cellular defense, detoxification, and immunity pathways (Hughes et al., 2011). The observed upregulation in wMelPop infected cell lines could be due to the difference in cell lines, but could also point to a strain-specific *Wolbachia* variation, suggesting that *Wolbachia*-induced effects on host immunity should not be generalized but determined for specific hosts.

Another recent study has suggested that reactive oxygen species (ROS), which are often involved in antimicrobial defense, may be associated with *Wolbachia* infection in an insect cell line (Brennan et al., 2008). Anti-oxidant proteins are often produced to detoxify ROS, and were observed to be upregulated in *Wolbachia*-infected cell lines. Furthermore, ROS production was observed to be greater in *Wolbachia*-infected cell lines and found to be associated with *Wolbachia*-infected vacuoles in host cell cytoplasm. Alternative hypotheses that have been suggested include the modification of host cell membranes, inhibiting particular pathogens from entering cells, or the competition between pathogens and *Wolbachia* for resources within cells (Moreira et al., 2009b).
Additional Wolbachia Phenotypes

In two recent publications, the wMelPop infection has also been shown to affect blood-feeding behavior in A. aegypti. Turley et al. (2009) demonstrated that infected females obtain fewer and smaller blood meals than Wolbachia-uninfected controls. In addition, the infected females displayed a behavioral characteristic that the authors describe as a ‘bendy’ proboscis that may help to explain the decrease in biting success. The behavioural modification was further described by using video recordings of feeding trials on a human hand to examine the pre-probing (time spent until inserting of mouthparts) and probing (time spent after insertion of mouthparts) behaviour of Wolbachia-infected compared to uninfected controls (Moreira et al., 2009a). Wolbachia-infected females spend a significantly longer time and an increased number of attempts at probing before the insertion of the mouthparts compared to uninfected controls. The effect was further exacerbated as the age of the mosquito increased. Furthermore, their results suggested that the number of Wolbachia-infected mosquitoes that successfully imbibed blood was reduced when compared to uninfected controls.

Symbiont interactions affecting pathogen transmission

While several recent bodies of work have focused specifically on the effects of Wolbachia, another has suggested an interactive effect of Wolbachia and other symbionts found in insects. In Aedes albopictus, the presence of Wolbachia and cultivable bacteria of the genera Acinetobacter, Comamonas, Delftia, and Pseudomonas suggest a potential interactive effect of other symbionts on the vector competence of insect hosts (Zouache et al., 2009). The interaction of three symbionts, Wolbachia, Wigglesworthia, and Sodalis in the African sleeping sickness vector, tsetse flies (Glossina spp.), may also play an important role in the vector competence of its host. Researchers led by Serap Aksoy at Yale University are currently investigating the role of Wolbachia and the other symbionts found in tsetse, and the implications of using the symbionts for a Wolbachia-based control strategy (Rio et al., 2004; Aksoy et al., 2008). Whether these symbiont interactions induce an immune or some alternative physiological response that inhibits pathogen transmission remains to be seen. Nevertheless, the presence of other symbionts or bacteria flora should not be ignored when investigating the effects of Wolbachia.

Releases of Wolbachia infected mosquitoes for vector control

Wolbachia effects on host fitness and the potential impact on control strategies The fitness of Wolbachia-infected males for incompatible insect technique (IIT) release strategies has been investigated as part of several recent studies in preparation for mosquito releases. An important consideration for an IIT strategy is that males are able to compete with naturally-occurring males. Brelsfoard and Dobson (In press), has examined the fitness of Aedes polynesiensis with an artificial infection type that is incompatible with naturally infected populations. Artificially infected strains were observed to have increased larval mortality and decreased adult longevity when compared to aposymbiotic strains (strains lacking Wolbachia and potentially other bacteria). Female fecundity and egg hatch rates of artificially and naturally infected and aposymbiotic strains were observed to be similar. Additionally, male age was not shown to influence rates of CI. The male mating competitiveness of A. polynesiensis strains with the artificial, incompatible infection type has also been examined in the laboratory (Brelsfoard et al., 2008) and in field cages (Chambers et al., 2011). In both studies, artificially infected males were equally competitive mates for naturally occurring males, suggesting the utility of this strain for an IIT strategy focused on controlling A. polynesiensis.

Calvitti et al. (2009) have also investigated the effects on male fitness of A. albopictus in response to removing natural Wolbachia infections. The removal of the natural Wolbachia infections is a necessary preliminary step before the introduction of a novel infection type. Results suggested that uninfected males had similar longevity and reproductive potential (mating competitiveness and sperm capacity) as naturally infected males. Following the examination of fitness of an aposymbiotic strain, a novel Wolbachia infection in A. albopictus has been developed via microinjection of wPip Wolbachia from C. pipiens into aposymbiotic eggs (Calvitti et al., 2010). The new infection type did not affect immature and adult survivorship, but did reduce female fecundity and egg hatch. While there were observed fitness reductions in females, maternal inheritance of Wolbachia and CI rates remained high when
artificially infected males were mated with naturally infected females. The results highlight the potential for the development of a Wolbachia-based IIT strategy for A. albopictus.

Wolbachia effects on host fitness may impact the spread of Wolbachia into a naive population as part of a population replacement strategy. Suh et al. (2009) demonstrated that wMelPop infections in A. albopictus can have deleterious effects on host fitness. In this case, wMelPop appears to act more as a pathogen by reducing fecundity and inducing a high rate of embryonic mortality. Furthermore, low rates of CI were observed in wMelPop-infected A. albopictus, suggesting that artificially introduced infections may not induce similar phenotypes as in other insect systems, and wMelPop infections may not be applicable for use as a biological control strategy for all insect systems. While the pathogenicity of the wMelPop strain was initially described as less severe in A. aegypti, its applied use has been abandoned in an initial Australian field trial in favor of the wMel infection (Hoffmann et al., 2011).

In a study by Yeap et al. (2011), CI and maternal inheritance rates, and effects on host fitness were examined in wMelPop-infected A. aegypti. CI and maternal inheritance rates were high, and adult longevity reduced. However, when immature mosquitoes were reared at high densities with limited food, wMelPop-infected adults were smaller when compared to uninfected adults. Therefore, larger uninfected males are able to deposit more sperm in females, increasing their chance of fertilization of wild-type females’ eggs, giving uninfected individuals a reproductive advantage. Also, the hatch rate of eggs that were deposited by wMelPop-infected females decreased as the eggs increased in age when compared to uninfected females. This observation could lead to a fitness disadvantage in situations where eggs need to persist before hatching, such as in dry conditions. Older wMelPop-infected females were also demonstrated to have reduced egg hatch rates when compared to uninfected females. This suggests again that infected females have a fitness disadvantage. The larval conditions in the field are variable and often with limited resources for developing larvae, suggesting that the spread of Wolbachia may be limited by larval density levels in oviposition sites and environmental conditions in the field that effect population dynamics.

Recently developed models have also suggested that not only CI but also the ecology and population dynamics of host insects can have major effects on the ability of Wolbachia infections to spread through populations (Hancock et al., 2011; Crain et al., In press). Further investigation of mosquito population dynamics and larval ecology of Wolbachia infected and uninfected mosquitoes in the laboratory and especially in a field setting are needed to determine and understand Wolbachia spread in natural populations.

Releases of Wolbachia infected insects Field releases of mosquitoes with artificial Wolbachia infections have been performed. Releases of Wolbachia-infected males to test the IIT have been undertaken on a small, isolated island in French Polynesia. While analysis is still pending, a decrease in population size at treatment sites and an increase in the proportion of non-hatching broods from captured females compared to control sites was observed, suggesting there was a population suppression effect associated with releases of incompatible males (O’Connor et al., 2010). These results are promising, but similarly to SIT strategies there is a requirement to rear and accurately separate males from females to avoid the risk of unintentional population replacement. Additionally, males need to be reared efficiently en masse, and remain competitive with wild-type males, an issue well recognized by previous SIT strategies (Alphey, 2002). Technologies to rear mosquitoes on a mass scale are needed and require further development.

Releases of Aedes aegypti that are infected with the wMel infection have recently been completed in Cairns, Australia, with additional small-scale releases planned for Vietnam and Thailand (Enserink, 2010; Christodoulou, 2011; Hoffmann et al., 2011; S. O’Neill, personal communication). The releases are part of an international research program led by Scott O’Neill at the University of Queensland. Releases consisted of approximately ten adult mosquitoes per household for ~10 weeks, with the ultimate goal of monitoring Wolbachia infection spread (Enserink, 2010; Hoffmann et al., 2011). The wMel infection was able to successfully invade two natural populations, reaching near fixation in a few months (Hoffmann et al., 2011). The releases are the first field studies to examine for the spread of an artificially introduced Wolbachia infection into a population.

Regulatory issues Releasing Wolbachia-infected insects for control of disease is not without any concerns for possible side effects on the human population and natural environment (Aultman et al., 2000). Popovic et al. (2010) has recently summarized several safety concerns of a Wolbachia-based control strategy for mosquitoes. The paper addresses questions concerning
the social engagement of the community in the release areas, concerns of the transfer of *Wolbachia* to humans, ethical issues, and the transfer of *Wolbachia* infections to the environment and/or natural predators using empirical studies. According to the studies presented in the paper, there was no experimental evidence that suggested any negative impact of *Wolbachia*-infected mosquitoes on humans, other organisms, or the environment.

*Wolbachia*-based strategies may be faced with less regulatory issues than genetic control-based strategies, if the *Wolbachia* and infected insects are not genetically modified. The releases in Australia were regulated and approved for use by the Australian Pesticides and Veterinary Medicines Authority. It was concluded that there was negligible risk and the releases would result in no more harm than that which is caused by the naturally uninfected populations of *A. aegypti* (Murphy et al., 2010). The releases in Australia provide an example for other countries to follow; however, regulations will need to be clearly defined by an internationally recognized agency before large-scale releases and or the commercialization of *Wolbachia*-based strategies for insect control. An additional topic of conversation for scientists is the issue of public acceptance of the release of large numbers of biting female mosquitoes. Greater than 140,000 adult mosquitoes were released at each site in Australia as part of the population replacement field trial (Hoffmann et al., 2011). Considerably larger releases may be required to obtain the same results on a larger scale or in areas with a higher density of mosquitoes than observed at the Australian release sites. The release number of biting female mosquitoes will need to be considered when designing and implementing *Wolbachia*-based strategies such as population replacement, and whether it will acceptable by the public.

**Conclusions**

Recent developments concerning the effects of *Wolbachia* infections on host pathogen transmission and the release of *Wolbachia*-infected mosquitoes highlights the potential of *Wolbachia* as an environmentally friendly biotechnology to control insect transmitted disease. Thus, determining the mechanisms responsible for pathogen inhibition will be important. Future research should focus on understanding the activation of the hosts’ immune response to *Wolbachia* infections and pathogens. Understanding the intracellular interaction of *Wolbachia* and the pathogen will aid in identifying any competitive effects for resources that exist within the cell. Furthermore, while the mosquito releases discussed have been on a small scale and have passed initial regulatory hurdles, it remains to be seen whether releases may be performed on a larger scale. Moreover, the release programmes have not examined the impact that releases may have on disease transmission. Future studies should also focus on the epidemiology of disease before and after the release of *Wolbachia*-infected insect vectors. Regardless of the lack of understanding of the underlying mechanisms of *Wolbachia*-induced pathogen inhibition, ongoing laboratory and field research promotes the study of and transfer of *Wolbachia* to other insect vectors of human pathogens, insect pests, and vectors of agricultural diseases.

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