EMBO reports

review

Wolbachia and the biological control of mosquito-borne disease

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Mosquito-borne diseases such as malaria, dengue fever and filariasis cause an enormous health burden to people living in tropical and subtropical regions of the world. Despite years of intense effort to control them, many of these diseases are increasing in prevalence, geographical distribution and severity, and options to control them are limited. The transinfection of mosquitos with the maternally inherited, endosymbiotic bacteria *Wolbachia* is a promising new biocontrol approach. Fruit fly *Wolbachia* strains can invade and sustain themselves in mosquito populations, reduce adult lifespan, affect mosquito reproduction and interfere with pathogen replication. *Wolbachia*-infected *Aedes aegypti* mosquitoes have been released in areas of Australia in which outbreaks of dengue fever occur, as a prelude to the application of this technology in dengue-endemic areas of south-east Asia.

Keywords: *Aedes aegypti*; Chikungunya; dengue; *Drosophila; Wolbachia pipientis*

EMBO *reports* advance online publication 6 May 2011; doi:10.1038/embor.2011.84

See Glossary for abbreviations used in this article.

Introduction

Insect-borne diseases, particularly those transmitted by mosquitoes, are among the leading causes of mortality and morbidity in humans. Malaria—caused by infection of *Plasmodium* protozoan parasites by the bite of anopheline mosquitoes—results in an estimated 1–2 million deaths per year, taking a dramatic toll on health and socio-economic development in affected areas (WHO, 2008). The annual incidences of mosquito-borne diseases including dengue fever, yellow fever, Japanese encephalitis, West Nile virus, Chikungunya and lymphatic filariasis are increasing due to human travel, rapid urbanization and failures of preventative public-health measures (Adams & Kapan, 2009; Chen & Wilson, 2010; Gould & Solomon, 2008; Gubler, 2002). Dengue fever is the most important arboviral disease in humans; 40% of the population of the world in more than 100 countries is at risk of infection and an estimated 50 million–100 million cases occur annually (Guzman & Kouri, 2002; Kyle & Harris, 2008; WHO, 2009). Dengue viruses (DENV) are primarily transmitted by the infectious bite of female *Aedes aegypti* mosquitoes and, to a much lesser extent, *Aedes albopictus* (Lambrechts *et al*, 2010). No effective vaccines or treatments against dengue fever exist (Wilder-Smith *et al*, 2010), and control methods are failing to prevent the global increase in the incidence of the disease (Morrison *et al*, 2008). New approaches are clearly needed if these trends are to be reversed.

The use of insecticides to target mosquitoes as a means of disease control can be effective, but is often prohibitively expensive, unsustainable and environmentally undesirable. This is particularly evident for anthropophilic species such as *A. aegypti*, which breed in densely populated urban and semi-urban areas (Wilder-Smith & Gubler, 2008). Furthermore, repeated exposure of mosquitoes to insecticides has allowed insecticide resistance to develop, increasing the need to use more-expensive alternative compounds (WHO, 1998; Zaim & Guillet, 2002). Insecticides are normally only used for the control of dengue fever during outbreaks, and their effectiveness is questionable. Alternative approaches aimed at environmental management of mosquitoes—such as the removal of oviposition sites, the introduction of mosquito predators such as fish or copepods (Kay & Vu, 2005) and personal protection against mosquito bites (repellents and nets)—have been beneficial in some cases. However, these strategies often require constant intervention, and can be expensive and difficult to implement in urban areas.

Wolbachia pipientis and disease control

The potential application of the symbiotic bacteria *Wolbachia pipientis* to the control of mosquito-borne diseases has emerged as a recent addition to the arsenal of weapons against mosquitoes. It has the benefit of being more environmentally benign than insecticide-based approaches and potentially more cost effective. *Wolbachia*-induced cytoplasmic incompatibility (CI) was proposed as a tool for *Culex* mosquito control as early as 1967 (Laven, 1967) and there were trials to eradicate mosquitoes in India in the 1970s (Curtis & Adak, 1974), but although there has been some field testing, it has never been operationally implemented. In recent years, there has been a resurgence of interest in *Wolbachia* as a means by which to control insect-transmitted diseases.

Wolbachia—an initially obscure α-proteobacterium, first identified in the ovaries of *Culex* mosquitoes in 1924 (Hertig & Wolbach, 1924)—is probably the most-common known endosymbiotic

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Received 28 February 2011; accepted 12 April 2011; published online 6 May 2011

Glossary

CI	cytoplasmic incompatibility
CHIKV	Chikungunya virus
CLA	cell-line adapted
DCV	*Drosophila* C virus
DENV	dengue virus
LRIM1	leucine-rich repeat immune protein
TEP1	thioester containing protein

microbe in the biosphere. It is thought to infect up to 76% of the estimated 2 million–5 million insect species on Earth (Hilgenboecker *et al*, 2008; Jeyaprakash & Hoy, 2000). The success of these small (0.5–1 μm), intracellular bacteria has been attributed to their ability to induce a series of reproductive distortions in their hosts to increase the reproductive success of infected females, thus enhancing the maternal transmission of *Wolbachia* (Werren *et al*, 2008). These traits include transforming genotypic males into phenotypic females, modifying male sperm so that females cannot produce progeny unless they mate with a male infected with the same strain of *Wolbachia*, or inducing the parthenogenetic reproduction of females (Stouthamer *et al*, 1999). *Wolbachia* can also provide direct fitness benefits to their hosts by affecting nutrition and development (Brownlie *et al*, 2009; Hosokawa *et al*, 2010), influencing fecundity (Aleksandrov *et al*, 2007) or oogenesis (Dedeine *et al*, 2001) and providing resistance to pathogens (Bian *et al*, 2010; Glaser & Meola, 2010; Hedges *et al*, 2008; Kambris *et al*, 2010; Moreira *et al*, 2009a; Osborne *et al*, 2009; Panteleev *et al*, 2007; Teixeira *et al*, 2008). Interestingly, some *Wolbachia* strains seem to have lost the ability to synchronize their replication with the host cell, and can dramatically reduce the lifespan of their hosts. One *Wolbachia* strain (*w*MelPop) that shortens the lifespan of adult *Drosophila* by up to 50% was discovered from experiments on fruit-fly lifespan mutants (Min & Benzer, 1997). This strain—also named 'popcorn', due to its ability to over-replicate and fill the brain tissues of infected flies—has been proposed as a potential tool for the control of mosquito-borne diseases, as it also reduces the longevity of adult female mosquitoes (Brownstein *et al*, 2003; McMeniman *et al*, 2009; Sinkins & O'Neill, 2000).

Although mosquito-borne pathogens can differ intrinsically—for example, malaria is caused by a protozoan parasite, whereas dengue fever is caused by a single-stranded RNA virus—their transmission is always influenced by the age of the mosquito. The reason for this is relatively simple: pathogens need to replicate in the body of the mosquito before reaching the salivary glands, in order to be successfully transmitted into a new human host by a bite. This period of development within the mosquito is called the extrinsic incubation period (EIP). Consequently, only female mosquitoes that are older than the EIP—usually 10–14 days for dengue fever (Salazar *et al*, 2007)—are vectors of epidemiological importance. Thus, disease-control approaches that aim to reduce mosquito lifespan have the potential to decrease disease transmission (Brownstein *et al*, 2003; Cook *et al*, 2008; Rasgon *et al*, 2003; Sinkins & O'Neill, 2000). Using *w*MelPop *Wolbachia* for disease control involves transferring this life-shortening *Wolbachia* strain into mosquito populations to remove the older individuals, which are the ones able to transmit the pathogen, from the population. The efficient maintenance and spread of the *Wolbachia* infection into field populations is crucial to the success of this strategy. Parallel approaches to reduce mosquito lifespan are also being developed, such as the use of spores from entomopathogenic fungi such as *Beauveria bassiana* in mosquito traps (Blanford *et al*, 2005; Darbro & Thomas, 2009; Thomas & Read, 2007) or delayed-acting insecticides (Read & Thomas, 2009).

Wolbachia transfer into *A. aegypti* mosquitoes

Although *Wolbachia* infections are relatively common in mosquitoes (Kittayapong *et al*, 2000; Ricci *et al*, 2002) including *Culex pipiens* (Yen & Barr, 1973), *C. quinquefasciatus*, *Aedes fluviatilis* (Moreira *et al*, 2009a) and *A. albopictus* (Sinkins *et al*, 1995), the main vectors for dengue fever (*A. aegypti*) and malaria (*Anopheles* spp.) are not naturally infected by *Wolbachia*. Approaches that use *Wolbachia* for the control of diseases transmitted by uninfected, naive insects rely on the successful establishment of stable *Wolbachia* infections, usually by embryonic microinjection of *Wolbachia*-infected cytoplasm or *Wolbachia* purified from infected insect hosts. To create stably transinfected lines, embryo injections must target the region near the pole cells in pre-blastoderm embryos to incorporate *Wolbachia* into the developing germline and favour the transmission of *Wolbachia* to offspring. Several *Wolbachia* strains have been transferred across sometimes phylogenetically distant insects (Table 1) and, importantly, the phenotypes induced by these strains in their native hosts are generally also expressed in the newly infected hosts.

Wolbachia transinfection experiments are more likely to be successful when the donor and recipient organisms are closely related. In line with this, the transfer of *w*MelPop from its natural host, *Drosophila melanogaster*, into the dengue fever vector *A. aegypti* was achieved in our laboratory after *Wolbachia* was first maintained by continuous passage in *A. albopictus in vitro* cell culture for almost 4 years (McMeniman *et al*, 2008; Fig 1). *Wolbachia* adapted to a mosquito intracellular environment, facilitating transfection *in vivo*. After microinjection of thousands of *A. aegypti* embryos, two stable *w*MelPop-CLA (cell-line-adapted) lines with maternal transmission rates of approximately 100% were generated (McMeniman *et al*, 2009; Fig 1). *w*MelPop-CLA-infected mosquitoes showed an approximately 50% reduction in adult lifespan, compared with their uninfected counterparts (McMeniman *et al*, 2009; Fig 2). The halving of adult mosquito lifespan and the high *Wolbachia* maternal transmission rates were also maintained in more genetically diverse outbred mosquitoes, and larval nutrition did not affect the life-shortening ability of the *w*MelPop-CLA strain (Yeap *et al*, 2010). The *w*MelPop-CLA infection is widespread in *A. aegypti* tissues, with high bacterial densities in the head (brain and ommatidia), thorax (salivary glands, muscle) and abdomen (fat tissue, reproductive tissues and Malphigian tubules; Moreira *et al*, 2009a; Fig 3). Wide distribution across tissues has been found in other transinfected mosquitoes, such as *A. aegypti* infected with the *w*AlbB strain from *A. albopictus* (Bian *et al*, 2010). By using quantitative PCR and western blot analyses, this strain was also found in reproductive tissues, midgut, muscles and heads, in both native *A. albopictus* (Dobson *et al*, 1999) and the transfected *A. aegypti* (Bian *et al*, 2010), although the densities are not as high as those found in *A. aegypti* infected with *w*MelPop-CLA.

Wolbachia interference with viruses and parasites

A key element in the use of *Wolbachia* for the control of insect-borne disease has been the discovery that some *Wolbachia* strains can interfere with insect viruses in *Drosophila* and human pathogens in mosquitoes. *Wolbachia* strains can protect *Drosophila* flies from RNA viruses such as *Drosophila* C (DCV), Cricket paralysis, Flock House and Nora viruses (Hedges *et al*, 2008; Osborne *et al*, 2009; Teixeira

Table 1 | *Wolbachia* strains transinfected into mosquitoes and induced phenotypes

Strain	Original host	Transinfected host	Phenotype in new host	Reference
*w*Mel	*Drosophila melanogaster*, then *Aedes albopictus* cell line	*Aedes aegypti*	CI, DENV interference	(T. Walker *et al*, unpublished data)
*w*MelPop-CLA	*Drosophila melanogaster*, then *Aedes albopictus* cell line	*Aedes aegypti*	CI, life shortening	(McMeniman *et al*, 2009)
			Blood-feeding alteration	(Turley *et al*, 2009)
			Bendy proboscis	(Moreira *et al*, 2009b)
			Plasmodium, DENV, CHIKV interference	(Moreira *et al*, 2009a)
			Increased metabolism	(Evans *et al*, 2009)
			Increased activity	(Kambris *et al*, 2009)
			Inhibition of filarial nematodes	
*w*AlbB	*Aedes albopictus*	*Aedes aegypti*	CI	(Xi *et al*, 2005)
			DENV interference	(Bian *et al*, 2010)
*w*AlbA, B	*Aedes albopictus*	*Aedes aegypti*	Partial CI	(Ruang-Areerate & Kittayapong, 2006)
*w*Ri	*Drosophila simulans*	*Aedes albopictus*	CI	(Xi *et al*, 2006)
*w*MelPop	*Drosophila melanogaster*	*Aedes albopictus*	CI, life shortening, embryo mortality	(Suh *et al*, 2009)
*w*Pip	*Culex pipiens*	*Aedes albopictus*	CI, lower hatch rate, reduced fecundity	(Calvitti *et al*, 2010)

CI, cytoplasmic incompatibility; CHIKV, Chikungunya virus; DENV, dengue virus.

et al, 2008), West Nile virus (Glaser & Meola, 2010), as well as the fungus *Beauveria bassiana* (Panteleev *et al*, 2007). Interestingly, the presence of *Wolbachia* interferes with a wider range of pathogens in transinfected mosquitoes including nematodes and bacteria (Kambris *et al*, 2009), viruses such as DENV (Fig 2,3F) and Chikungunya (CHIKV; Bian *et al*, 2010; Moreira *et al*, 2009a), as well as the avian and rodent malaria parasites *Plasmodium gallinaceum* (Moreira *et al*, 2009a) and *P. berghei* (Kambris *et al*, 2010). Natural *Wolbachia* strains that infect mosquitoes have also been shown to induce resistance to viruses—as in *C. quinquefasciatus* mosquitoes, that are resistant to West Nile virus (Glaser & Meola, 2010)—although this resistance seems less pronounced in comparison to transinfected *Wolbachia* strains such as *w*MelPop-CLA (Moreira *et al*, 2009a).

The mechanisms by which some *Wolbachia* strains interfere with a variety of pathogens remain unclear. One hypothesis is that pathogen interference is partly mediated by the induction of antimicrobial peptides and pre-activation of the innate immune response in the insect (Kambris *et al*, 2010; Kambris *et al*, 2009; Moreira *et al*, 2009a). The presence of *w*MelPop-CLA *Wolbachia* in *A. aegypti* induced the expression of several immune effector molecules, including cecropin, defensin, thio-ester containing proteins and C-type lectins (Moreira *et al*, 2009a). When the *w*MelPop strain was transiently injected into adult *Anopheles gambiae*, several immune genes were upregulated, as shown by whole-genome arrays (Kambris *et al*, 2009), resulting in the inhibition of *Plasmodium* development (Kambris *et al*, 2010). Some of these genes, in particular *LRIM1* and *TEP1*, have been found to inhibit *Plasmodium* development by interfering with the opsonization pathway (Blandin *et al*, 2004; Povelones *et al*, 2009). Indirect supporting evidence for the role of the immune system in pathogen suppression was reported by Kokoza and colleagues, who showed that the co-expression of two antimicrobial peptides (cecropin A and defensin A) in transgenic *A. aegypti* induces resistance to infection with the bacterial pathogen *Pseudomonas aeruginosa*, and reduces the number of *P. gallinaceum* oocysts, completely blocking transmission of this avian malaria parasite to naive chickens (Kokoza *et al*, 2010). Furthermore, *Wolbachia* infections in *A. aegypti* and *Drosophila* have been shown to increase haemolymph melanization (Thomas *et al*, 2010), a key constituent of the insect innate immune system that is involved in the encapsulation of foreign bodies and parasites (Carton & Nappi, 1997; Theopold *et al*, 2004).

Wolbachia-mediated pathogen interference is unlikely to result solely from an upregulation of the insect host immune system. *Wolbachia* infection provides protection against DENV in mosquito cell lines, even though these cells lack whole-organism or tissue-specific immunity (Frentiu *et al*, 2010). Similarly, the *Wolbachia* strain *w*Ri does not induce an immune response in its native *D. simulans* hosts (Bourtzis *et al*, 2000), and yet confers protection against DCV in these flies (Osborne *et al*, 2009). However, *w*Ri induces an immune response in transinfected *D. melanogaster* cell lines (Xi *et al*, 2008), and mosquitoes artificially infected with *Wolbachia* also show upregulation of the immune system (Kambris *et al*, 2010; Kambris *et al*, 2009; Moreira *et al*, 2009a). Given that immune response to *Wolbachia* infection has been observed only in hosts artificially infected with new strains, such responses might not be due to the *Wolbachia* infection, but instead to an unusual effect of the new host–symbiont combination, such as elevated, unnatural *Wolbachia* densities (McGraw *et al*, 2002).

The observed interference of some *Wolbachia* strains with DENV replication and dissemination could also be caused by direct competition for cellular resources. This seems to be a plausible explanation given (i) the high density of the *w*MelPop-CLA *Wolbachia* infection in most *A. aegypti* tissues, such as muscle, fat, nervous tissue, salivary glands and Malpighian tubules (Fig 3G); (ii) the exclusion between *Wolbachia* and DENV in the fat tissue of some mosquitoes that contain both microorganisms (Moreira *et al*, 2009a; Fig 3F); and (iii) the reliance of DENV replication on host fatty-acid synthesis (Heaton *et al*, 2010; Heaton & Randall, 2010), as fatty acids might be sequestered by *Wolbachia*. In agreement with this hypothesis, *Wolbachia*-mediated viral interference seems to depend on bacterial loads. In

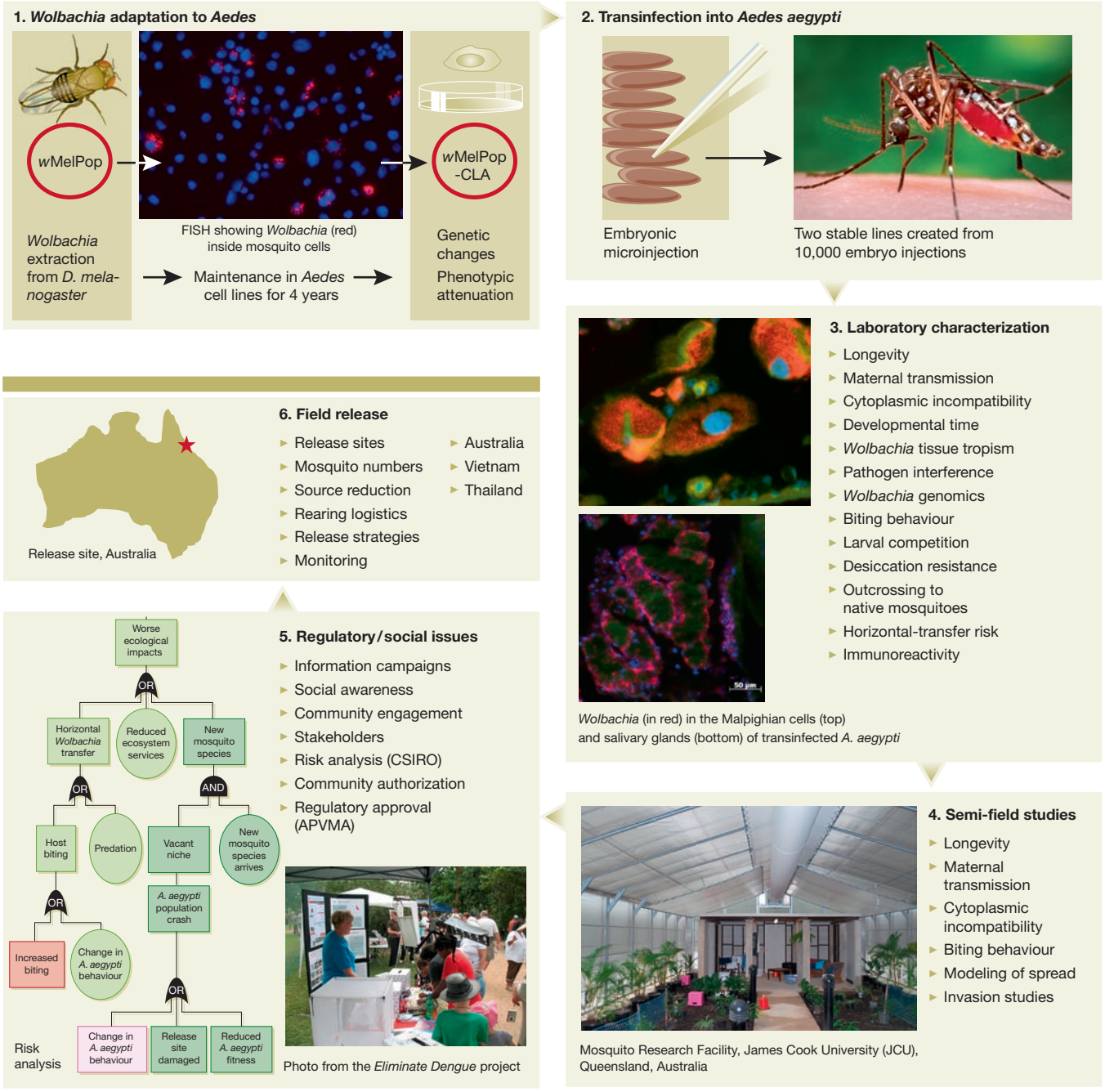

Fig 1 | From the generation of *Wolbachia*-infected mosquitoes to their release for the control of dengue fever in Australia. (**1**) *w*MelPop *Wolbachia* were extracted from their native *D. melanogaster* host and maintained in *Aedes* cell lines for 4 years. During this time, genetic changes and phenotypic adaptation occurred (McMeniman *et al*, 2008; I. Iturbe-Ormaetxe and J. Brownlie, unpublished data). (**2**) After injecting more than 10,000 *A. aegypti* embryos with purified *Wolbachia*, two stable transinfected mosquito lines were generated (McMeniman *et al*, 2009). (**3**) Transinfected mosquitoes were characterized for *Wolbachia*-induced phenotypes, including ability to block pathogen replication (Moreira *et al*, 2009a). (**4**) Studies were conducted in secure cages at James Cook University, Cairns, Australia, to determine the ability of transinfected mosquitoes to infect natural populations under semi-natural conditions. (**5**) Extensive community engagement and information campaigns (McNaughton *et al*, 2010), together with risk analysis (Murphy *et al*, 2010) preceded the granting of regulatory approval from the Australian government for the open release of the mosquitoes into the environment. (**6**) The release of *A. aegypti* mosquitoes transinfected with *w*Mel *Wolbachia* started in January 2011 in two townships near to Cairns, Queensland, Australia and will take place for 12 weeks. This will be followed by extensive monitoring of the invasion of *Wolbachia* into field populations of *A. aegypti*, and the effect of this on dengue-fever transmission, before further releases in Vietnam (Jeffery *et al*, 2009) and Thailand. APVMA, Australian Pesticides and Veterinary Medicines Authority; CSIRO, Commonwealth Scientific and Industrial Research Organisation.

Drosophila, strains that grow at higher densities such as *w*Mel and *w*Ri provide high levels of protection, whereas low-density strains such as *w*No and *w*Ha provide no protection against insect viruses (Osborne *et al*, 2009). Furthermore, transinfected *Wolbachia* strains in *A. aegypti* mosquitoes that grow to high densities—such as *w*MelPop-CLA—provide almost complete protection from pathogens (Moreira *et al*, 2009a). Variability in tissue density and localization is common across strains, and probably influences the effects on the host, as well as pathogen interference. For example, some *Aedes* species such as *A. fluviatilis* and *A. albopictus* can transmit *Plasmodium* and DENV/CHIKV, respectively, despite being naturally infected with *Wolbachia,* although they are not typically important epidemic vectors (Lambrechts *et al*, 2010). *A. fluviatilis* is infected with the *w*Flu strain of *Wolbachia* (Moreira *et al*, 2009a), but the tissue distribution and density of this strain is limited compared with the *w*Mel/*w*MelPop-CLA infections, showing more similarity to the superinfected *Wolbachia* strains present in *A. albopictus* (I. Iturbe-Ormaetxe, unpublished data). Lower *Wolbachia* densities in these species or a preference of these *Wolbachia* strains for reproductive tissues instead of gut, salivary glands or fat tissue—which DENV or *Plasmodium* parasites infect (Moreira *et al*, 2009a)—could explain the differences in vector competence. *Wolbachia* can also coexist with Japanese encephalitis viruses in *Armigeres* mosquitoes (Tsai *et al*, 2006), but whether this coexistence is related to density or tissue tropism is yet to be determined. The specific tissues that *Wolbachia* must infect and the role of tissue-specific *Wolbachia* density in pathogen interference also remain unknown.

Molecular genetics of *Wolbachia*

The molecular mechanisms underlying the reproductive phenotypes and pathogen interference of *Wolbachia* remain mostly unknown. Particular effort has been devoted to elucidating the mechanisms for CI, the main driving force for *Wolbachia* population invasion in transinfected mosquitoes. Among the effectors proposed to induce CI in *Drosophila* and mosquitoes, ankyrin (ANK) domain proteins have received special attention (Duron *et al*, 2007; Iturbe-Ormaetxe *et al*, 2005; Sinkins *et al*, 2005; Walker *et al*, 2007). Despite being relatively rare in prokaryotes (Sedgwick & Smerdon, 1999), ANK genes are particularly abundant in *Wolbachia* genomes (Klasson *et al*, 2009b; Walker *et al*, 2007; Wu *et al*, 2004) and are known to be involved in protein–protein interactions in many systems, including closely related *Anaplasma* spp. (Caturegli *et al*, 2000; Ijdo *et al*, 2007; Park *et al*, 2004) and *Ehrlichia* spp. (Rikihisa & Lin, 2010; Zhu *et al*, 2009). ANK repeats are found in proteins involved in cell-cycle regulation, in transcriptional regulators, toxins and cyclin-dependent kinase inhibitors (Al-Khodor *et al*, 2010; Bork, 1993; Michaely & Bennett, 1992; Sedgwick & Smerdon, 1999). Orthologue ANK proteins from closely related *Wolbachia* strains that induce different phenotypes in their hosts can vary greatly in their architecture, number of ANK repeats and presence of transmembrane domains. They can also be absent or truncated in some strains (Iturbe-Ormaetxe *et al*, 2005). This variability probably affects the affinity, specificity, localization, expression and function of these ANK proteins and makes them good candidates to be mediators in reproductive phenotypes. *Wolbachia* is an unculturable, untransformable bacterium and Yamada and colleagues therefore used an alternative approach to genetically test several candidate *Wolbachia* genes (Yamada *et al*, 2011). Transgenic *Drosophila* flies were created that expressed either a single candidate *Wolbachia* ANK gene or complemented these genes in *Drosophila* strains that were infected with a non-CI-inducing *Wolbachia* strain lacking the ANK gene to be tested. Both the expression of single ANK genes in *Wolbachia*-uninfected flies and the complementation of a single ANK gene in *Drosophila* infected with a non-CI *Wolbachia* strain were unable to mimic the CI phenotype, indicating that the expression of single ANK genes is not enough to induce CI, and additional factors must be involved (Yamada *et al*, 2011).

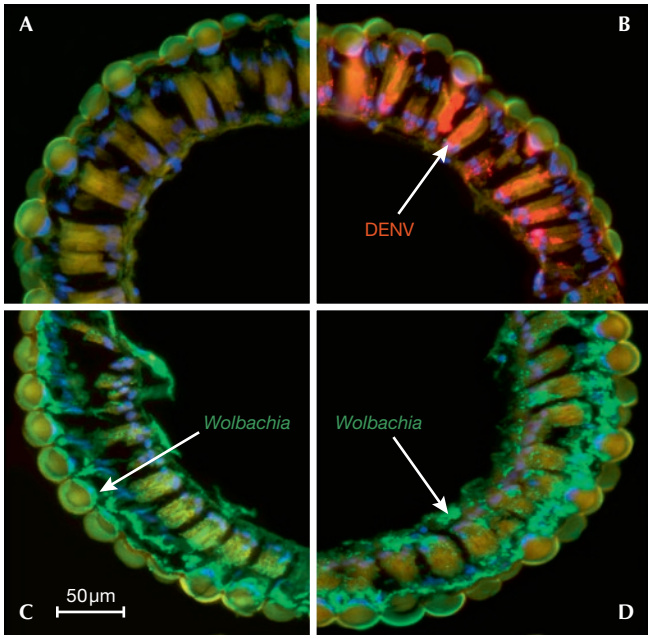

Fig 2 | Immunofluorescence staining of *Aedes aegypti* mosquito compound eyes (ommatidia). *w*MelPop-CLA *Wolbachia* is shown in green, DENV in red and DNA in blue. (**A**) Ommatidia section of a control, uninfected mosquito (no DENV, no *Wolbachia*). (**B**) Ommatidia of a *Wolbachia*-free mosquito, 14 days post-infection with DENV (seen in red). (**C**) Ommatidia of a *Wolbachia*-transinfected *A. aegypti* mosquito. Scale bar, 50 µm. (**D**) Ommatidia of a *Wolbachia*-transinfected *A. aegypti* mosquito, 14 days post-infection with DENV; DENV levels are dramatically reduced by the presence of *Wolbachia* and no DENV signal is detectable (Figure modified from Moreira *et al*, 2009a, with permission). CLA, cell-line adapted; DENV, Dengue virus.

The WO *Wolbachia* bacteriophage, which infects up to 90% of insect *Wolbachia* strains (Bordenstein & Wernegreen, 2004; Gavotte *et al*, 2004) has also been suggested to induce CI in insects, particularly as viral filtrates obtained from *Wolbachia* extracts are able to induce CI in *Nasonia* wasps (Williams *et al*, 1993). A series of WO-encoded genes, such as the virulence factor *VrlC*, have been implicated in *Wolbachia* pathogenicity (Kent & Bordenstein, 2010), due to the correlation between sequence variation in these genes and the induction of CI in *C. pipiens* mosquitoes (Duron *et al*, 2006). However, no correlation was found between the presence of phage capsid genes and the induction of CI in *Culex* mosquitoes (Gavotte *et al*, 2007). Bordenstein and colleagues proposed a model that links high *Wolbachia*-phage density with lower *Wolbachia* density and less CI (Bordenstein *et al*, 2006). At the cellular level, CI has been studied in *Drosophila* and the wasp *Nasonia* (Serbus *et al*, 2008),

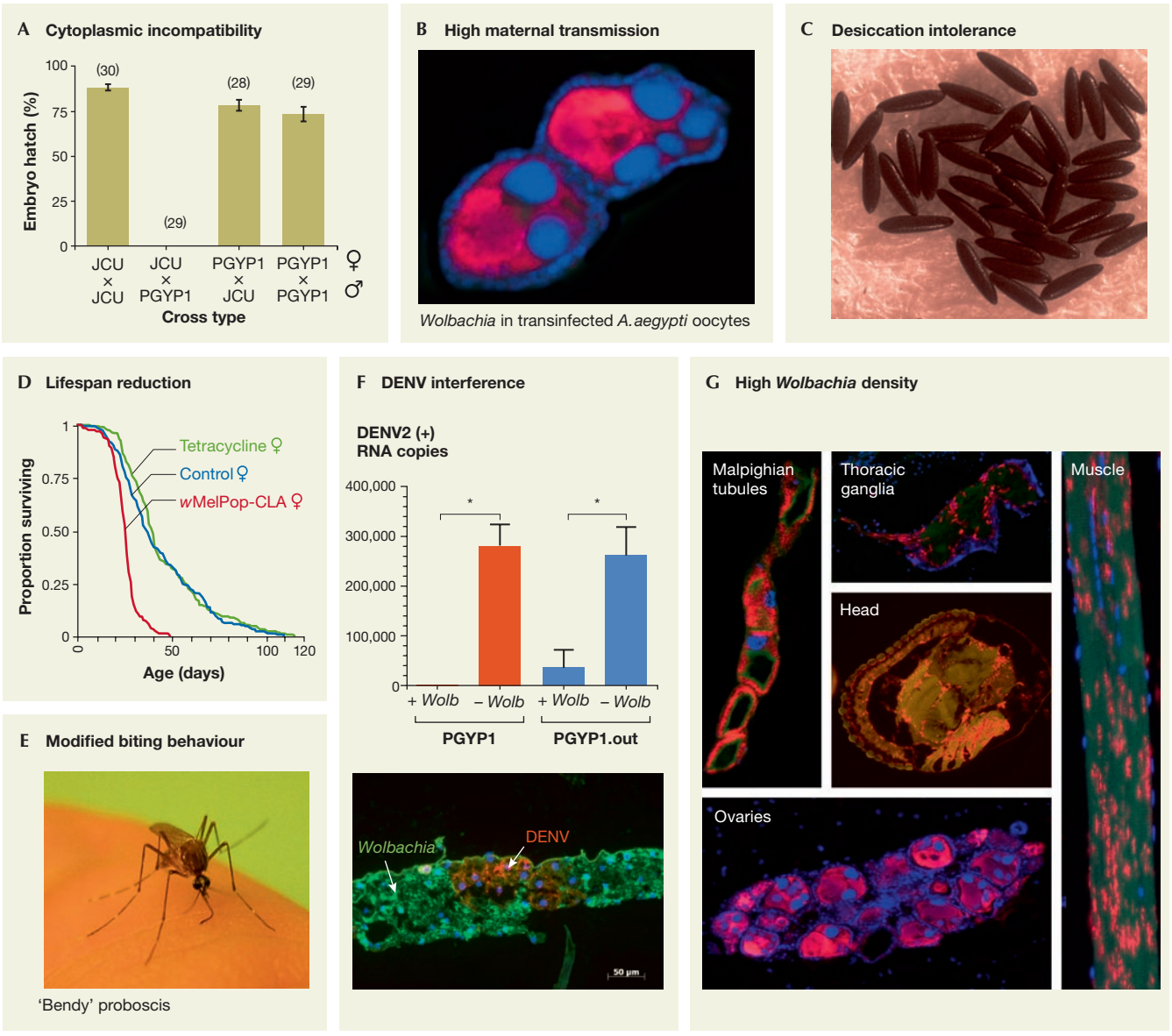

Fig 3 | Phenotypes induced by the *w*MelPop-CLA strain in transinfected *Aepes aegypti* mosquitoes. (**A**) 100% cytoplasmic incompatibility. Crosses between *Wolbachia*-infected males (PGYP1) and uninfected females (JCU) produce no offspring, whereas every other crossing combination produces viable embryos (Figure taken from McMeniman *et al*, 2009, with permission). (**B**) Transmission to almost 100% of the mosquito offspring, as suggested by the high levels of *Wolbachia* (in red) in the oocytes of infected females, shown by FISH (McMeniman *et al*, 2009; Moreira *et al*, 2009a). (**C**) Dramatic reduction of *A. aegypti* egg viability (McMeniman *et al*, 2011). (**D**) Reduction of adult mosquito lifespan by 50%, compared with wild-type mosquitoes or uninfected counterparts after tetracycline curing of the *Wolbachia* infection (Figure taken from McMeniman *et al*, 2009, with permission). (**E**) Alteration of biting behaviour and reduction of blood-feeding success in ageing *A. aegypti* mosquitoes. 'Shaky' and 'bendy' proboscis phenotypes are commonly observed (Turley *et al*, 2009; Moreira *et al*, 2009b). (**F**) Inhibition of DENV replication, in both inbred (PGYP1) and outbred (PGYP1.out) mosquitoes. The bottom panel shows the cellular exclusion of *Wolbachia* and DENV in fat tissue of some *Wolbachia*-infected mosquitoes 14 days post-DENV injection (Figures taken from Moreira *et al*, 2009a, with permission). (**G**) Widespread tissue infection in *A. aegypti*, including Malpighian tubules, thoracic ganglia, thoracic muscle, ovaries, heads and salivary glands (Fig 1) as shown by FISH (*Wolbachia* stained in red, DNA stained in blue; Moreira *et al*, 2009a; T. Walker *et al*, unpublished data). CLA, cell-line adapted; DENV, Dengue virus; FISH, flourescence *in situ* hybridization.

in which inhibition of Cdk1 activation, and chromosome segregation and condensation defects lead to delayed nuclear-envelope breakdown (Tram *et al*, 2003; Tram & Sullivan, 2002). *Wolbachia* also impairs H3.3/H4 histone accumulation in the male pronucleus, which ultimately leads to defects in transcription regulation in the host nuclei (Landmann *et al*, 2009).

However, determining the molecular basis of these phenotypic effects has been hampered by two difficulties: the obligate

symbiotic nature of *Wolbachia* makes it impossible to grow these bacteria in cell-free culture, and there is no genetic-transformation technology available that would make genetic testing possible. On the other hand, the sequencing of several complete *Wolbachia* genomes (Foster *et al*, 2005; Klasson *et al*, 2009a; Klasson *et al*, 2008; Salzberg *et al*, 2005; Salzberg *et al*, 2009; Wu *et al*, 2004) has been an important advance in the field, and several more are being sequenced or annotated (Iturbe-Ormaetxe *et al*, 2011; Werren *et al*, 2008).

Comparative genomic studies between the closely related *w*Mel (Wu *et al*, 2004) and *w*MelPop genomes revealed a series of genetic differences, including an approximately 150 Kb inversion, several insertions/deletions (including some ANK repeats and mobile elements), and about 200 single nucleotide polymorphisms, which might be responsible for the over-replication phenotype and the ability of *w*MelPop to shorten lifespan (Riegler *et al*, 2005; I. Iturbe-Ormaetxe and J. Brownlie, unpublished data). Interestingly, when the *w*MelPop strain was sequenced almost 4 years after adaptation to mosquito cells (*w*MelPop-CLA), we found that the genome had quickly evolved during cell culture through a series of deletion and insertion events that could be sequentially characterized over time (McMeniman *et al*, 2008; McMeniman *et al*, 2009; I. Iturbe-Ormaetxe and J. Brownlie, unpublished data). As a result, several *w*MelPop-CLA genes were inactivated, compared with the original *w*MelPop strain from flies. These genetic differences could explain the attenuation of the over-replication and the life-shortening phenotype that was observed after the *w*MelPop-CLA strain was transferred back into flies following its maintenance in cell culture (McMeniman *et al*, 2008). We are sequencing other closely related strains to identify the genetic basis for these phenotypes, in particular the over-replication and life-shortening phenotypes. However, the aforementioned lack of genetic-transformation technologies for *Wolbachia* will make functional genetic studies challenging.

Wolbachia invasion of mosquito populations

Wolbachia-infected female mosquitoes must transmit the bacteria to their progeny at a high frequency for it to spread successfully and invade uninfected insect populations. The *w*MelPop-CLA strain is transmitted from mother to offspring at almost 100% efficiency in transinfected *A. aegypti* mosquitoes (McMeniman *et al*, 2009). A nearly perfect maternal transmission rate is predicted from fluorescence *in situ* hybridization and immunolocalization studies, which show extremely high *Wolbachia* densities in ovaries of *w*MelPop-CLA-infected female mosquitoes (Fig 3B), ensuring the transfer of the infection to the next generation. Males are a dead end for *Wolbachia*, which is completely absent from mature sperm and seminal fluids.

Another important requirement is that *Wolbachia* spreads into uninfected populations through the induction of reproductive distortions that favour infected females. The *w*MelPop-CLA strain induces very high (almost 100%) CI rates in transinfected *A. aegypti* mosquitoes (Fig 3A; McMeniman *et al*, 2009). Maternal transmission and CI give infected females a reproductive advantage, as uninfected females produce no offspring when they mate with infected males. The ability of *Wolbachia*-infected *A. aegypti* to spread in semi-natural conditions has been tested in secure, outdoor mosquito cages in Australia (Fig 1.4; Ritchie *et al*, 2011); *Wolbachia* can spread to high frequencies approaching fixation in interbreeding, caged populations of mosquitoes in 3 months, when initial frequencies are two *Wolbachia*-infected mosquitos for every wild-type mosquito (T. Walker *et al*, unpublished data).

Fitness of *Wolbachia*-infected mosquitoes

Wolbachia-infected mosquitoes can only spread and invade uninfected mosquito populations if the fitness cost of infection is less than the fitness advantage that CI provides for the infection to spread. Pathogen protection might also provide a fitness advantage to *Wolbachia*-infected mosquitoes that will assist their spread in the field. Apart from the reduction in lifespan, some of the fitness effects induced by the *w*MelPop-CLA infection in *A. aegypti* include an increase of metabolic rate and activity in the mosquito (Evans *et al*, 2009), and a fecundity cost. The latter is detected as a steady reduction in hatch rates after the first gonothropic cycle, probably due to an impaired ability to feed as the mosquitoes age (Turley *et al*, 2009). Another significant effect of *w*MelPop-CLA infection in *A. aegypti* is the reduction of egg survival during periods of embryonic quiescence (Fig 3; McMeniman & O'Neill, 2010). This might be a desired control mechanism for population suppression in areas with pronounced wet/dry seasonality, by preventing the next generation of mosquitoes from hatching after the dry season.

The ability of mosquitoes infected with *w*MelPop-CLA to feed on human hosts has been tested by looking at the volume of blood they have ingested, their ability to probe successfully, and other aspects of their biting behaviour (Moreira *et al*, 2009b; Turley *et al*, 2009). *Wolbachia* does not affect the response time of mosquitoes to humans, but its presence reduces the number and size of blood meals taken. *w*MelPop-CLA *Wolbachia* also induced behavioural changes in old mosquitoes termed 'shaky' or 'bendy', in which the proboscis bends and is unable to pierce the skin; 65% of 35-day-old insects showed the bendy phenotype (Fig 3E; Turley *et al*, 2009). *Wolbachia*-infected *A. aegypti* produce smaller volumes of saliva, which contain the same levels of the anti-platelet-aggregation enzyme, apyrase, as uninfected mosquitoes (Moreira *et al*, 2009b).

Despite the ability of the *w*MelPop-CLA strain to induce strong CI and interfere with DENV replication in transinfected *A. aegypti* mosquitoes, the fitness effects produced in its host might be counterproductive to, or even completely block, the establishment of this strain in natural populations of mosquitoes (Turelli, 2010). Alternative, less-virulent strains might therefore be required.

In *Drosophila*, viral interference is induced by several *Wolbachia* strains that are closely related to *w*MelPop (Hedges et al, 2008; Osborne et al, 2009) suggesting non-life-shortening strains with more desirable invasion characteristics would also affect transmission of dengue fever. For example, the *w*Mel strain naturally infects wild populations of *D. melanogaster* worldwide (Riegler *et al*, 2005), suggesting a high potential for it to invade mosquito populations. We have recently established *w*Mel-infected *A. aegypti* lines that have lower fitness costs, but still significantly interfere with dengue (T. Walker *et al*, unpublished data). Strains such as this might be preferable to the *w*MelPop-CLA strain for deployment in the field.

From the lab to the field

The phenotypic effects of *Wolbachia* on laboratory *A. aegypti* colonies need to be tested in natural conditions and in mosquitoes with the same genetic backgrounds as those in the intended release area. Out-crossing laboratory-reared *w*MelPop-CLA-infected mosquitoes with Australian wild-stocks has revealed that the dengue-fever interference, life-shortening and fitness indicators are maintained (Moreira *et al*, 2009a; Yeap *et al*, 2010). Large, semi-natural,

purpose-built cages are being used to determine the ability of *Wolbachia* strains to invade uninfected mosquito populations.

A trial release of *Wolbachia*-infected *A. aegypti* is taking place in two localities in north Queensland, Australia, during the 2011 wet season, with regulatory approval from the Australian government and strong support from the community (Fig 1; McNaughton *et al*, 2010). Risk assessments have concluded that there is a negligible risk of the release of *Wolbachia*-infected *A. aegypti* resulting in more harm than that caused by naturally occurring *A. aegypti* over a 30-year period (Murphy *et al*, 2010). We have also addressed several safety concerns about releasing *Wolbachia*-infected mosquitoes into the field. There is no evidence for the transfer of *Wolbachia* to humans by mosquito bites (Popovici *et al*, 2010). Lateral transfer of *Wolbachia* to non-target species is unlikely as *Wolbachia* are maternally transmitted, and horizontal transfer has only been reported on rare occasions (Heath *et al*, 1999; Huigens *et al*, 2004). There is also no evidence of *Wolbachia* transfer to mosquito predators such as spiders and geckos (Popovici *et al*, 2010). There is potential for horizontal transfer of *Wolbachia* DNA into mosquito genomes (Fenn *et al*, 2006; Hotopp *et al*, 2007; Klasson *et al*, 2009a; Kondo *et al*, 2002; McNulty *et al*, 2010; Nikoh *et al*, 2008; Woolfit *et al*, 2009), but such cases take place over evolutionary timescales and are extremely rare. Furthermore, the consequences of such transfer are unlikely to increase the risk of adverse events associated with the open release of *Wolbachia*-infected mosquitoes.

Future perspectives

The ability of some *Wolbachia* strains to reduce the lifespan of *A. aegypti*, invade mosquito populations through the induction of CI and, in particular, interfere with the replication of a variety of pathogens, has placed this bacterium at the frontline of new approaches targeting mosquito-borne diseases in an environmentally friendly manner. The release of *Wolbachia*-infected, dengue-refractory mosquitoes in Australia in 2011 will create the basis for the implementation of this strategy in Vietnam, Thailand and possibly other countries in which the magnitude of dengue fever is higher and the ecology and breeding habits of mosquitoes—particularly in large urban areas—make control challenging. This *Wolbachia*-based, biocontrol approach could be applied to invasive mosquito species, such as *A. albopictus,* which has recently become a secondary dengue fever vector in Asia and has rapidly spread in the USA and large parts of Europe and Africa (Knudsen *et al*, 1996; Lambrechts *et al*, 2010). *A. albopictus* is also a vector for CHIKV in these countries (Bonilauri *et al*, 2008; Pages *et al*, 2009).

One main goal for the *Wolbachia*-based biocontrol approach to mosquito-borne-disease control is to transfer *Wolbachia* into anopheline mosquitoes, the most-common vectors of human malaria. The ability to maintain *Wolbachia* in *Anopheles* cell lines (McMeniman *et al*, 2008; Rasgon *et al*, 2006a; Rasgon *et al*, 2006b) and the successful establishment of transient somatic *w*MelPop infections in *Anopheles* mosquitoes (Jin *et al*, 2009; Kambris *et al*, 2010)—which inhibit *Plasmodium* development by activating the mosquito immune response (Kambris *et al*, 2010)—are promising advances that suggest stable transinfection might be possible.

A full list of *Wolbachia* literature and resources can be found at the *Wolbachia* website (http://www.wolbachia.sols.uq.edu.au) and information about the field release of *Wolbachia*-infected mosquitoes for dengue fever control can be found at (http://www.eliminatedengue.com).

ACKNOWLEDGMENTS

We thank L. Moreira for helpful and constructive comments on the manuscript. This work was supported by grants from the Foundation for the National Institutes of Health through the Grand Challenges in Global Health Initiative of the Bill & Melinda Gates Foundation, the National Health and Medical Research Council of Australia, the Australian Research Council and the National and International Research Alliances programme of the Queensland state government.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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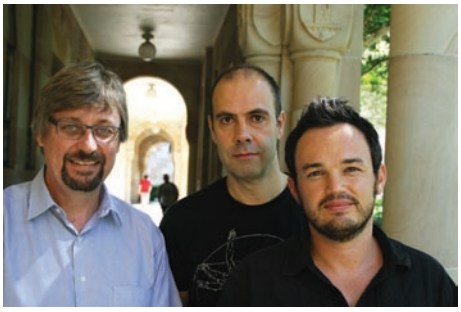

Scott L. O' Neill (left), Iñaki Iturbe-Ormaetxe & Thomas Walker (right)