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Review

Understanding the *Wolbachia*-mediated inhibition of arboviruses in mosquitoes: progress and challenges

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Arthropod-borne viruses (arboviruses) pose a considerable threat to human and animal health, yet effective control measures have proven difficult to implement, and novel means of controlling their replication in arthropod vectors, such as mosquitoes, are urgently required. One of the most exciting approaches to emerge from research on arthropods is the use of the endosymbiotic intracellular bacterium *Wolbachia* to control arbovirus transmission from mosquito to vertebrate. These α-proteobacteria propagate through insects, in part through modulation of host reproduction, thus ensuring spread through species and maintenance in nature. Since it was discovered that *Wolbachia* endosymbiosis inhibits insect virus replication in *Drosophila* species, these bacteria have also been shown to inhibit arbovirus replication and spread in mosquitoes. Importantly, it is not clear how these antiviral effects are mediated. This review will summarize recent work and discuss determinants of antiviral effectiveness that may differ between individual *Wolbachia*/vector/arbovirus interactions. We will also discuss the application of this approach to field settings and the associated risks.

Introduction

Arthropod-borne viruses (arboviruses) are transmitted in nature between susceptible vertebrate hosts and blood-feeding arthropod vectors, such as mosquitoes, sandflies, midges and ticks. Upon infection with arboviruses, vertebrate hosts can develop a range of symptoms from mild to severe, including encephalitis and haemorrhagic fever. Arthropod vectors acquire a lifelong, generally asymptomatic infection through feeding on viraemic vertebrates. Following the initial replication in midgut or hindgut epithelial cells, virus is released into the haemocoel from where it disseminates to other tissues, most importantly the salivary glands and ducts. The transmission cycle is completed when arboviruses present in the arthropod's saliva are passed to a vertebrate host during a subsequent bloodmeal. Arbovirus distribution is dependent on the presence of vector species in a given area. Changes in climate and vector distribution, and an increase in urbanization, human travel and livestock movements, can all potentially impact on arbovirus transmission (Weaver & Reisen, 2010). Most arboviruses are grouped into four RNA virus families, and include the positive strand *Togaviridae* and *Flaviviridae*, the negative strand segmented *Bunyaviridae* and the dsRNA segmented *Reoviridae* (Weaver & Reisen, 2010).

Control of arboviral disease may be achieved by a number of measures, such as vaccination or the development of antiviral drugs; however, these are often not available and/or prove difficult to develop. For example, there is no effective vaccine for dengue virus (DENV; *Flaviviridae*), which impacts significantly on human health worldwide (Halstead, 2012) or emerging arboviruses, such as the alphavirus chikungunya (CHIKV; *Togaviridae*).

Indeed, preventing transmission from arthropod vector to vertebrate, and thus preventing infection altogether, may present a valuable alternative in combating arboviral disease. Vector eradication programmes form part of such strategies. In the past, sterile mosquitoes have been released, and more recently, genetic modification techniques have been used successfully to control mosquito populations (Alphey *et al.*, 2013; Black *et al.*, 2011; Lacroix *et al.*, 2012; Wilke & Marrelli, 2012). However, because one approach alone is unlikely to be sufficient and/or always applicable, novel strategies that work at the level of vector control are required.

In this context, current efforts to manipulate transmission of arboviruses by mosquitoes through the use of the bacterium *Wolbachia* merit greater attention. As RNA virus replication is inhibited by these endosymbionts in the model arthropod *Drosophila melanogaster* (Hedges *et al.*, 2008; Teixeira *et al.*, 2008), *Wolbachia* has also been used to control arbovirus replication in mosquitoes and their transmission to vertebrates.

Here we summarize recent research focusing on *Wolbachia*/mosquito/arbovirus interactions and discuss progress into the mechanisms by which these endosymbionts confer antiviral resistance to their hosts, as well as perspectives for further research.

Wolbachia and reproductive phenotypes

Wolbachia is a genus of intracellular α-proteobacteria (family *Rickettsiaceae*), which is thought to infect 40–75 % of all arthropod species and several nematodes (Hilgenboecker *et al.*, 2008; Zug & Hammerstein, 2012). First discovered in the mosquito *Culex pipiens* (Hertig & Wolbach, 1924), these bacteria have now been found in a number of vector mosquito species, although not the major arbovirus vector *Aedes aegypti* (Kittayapong *et al.*, 2000; Sinkins, 2004).

Wolbachia has been extensively studied because of its ability to significantly alter the biology of the host in the symbiotic relationship. These alterations are thought to dramatically increase the spread of the bacteria through insect populations and include male killing, parthenogenesis, feminization and cytoplasmic incompatibility (CI) (Werren *et al.*, 2008). In mosquito populations, *Wolbachia* is spread and maintained through unidirectional and bidirectional CI (Kambhampati *et al.*, 1993; Sinkins, 2004; Yen & Barr, 1973), and our understanding of this process is vital to successful introduction of *Wolbachia*-infected mosquitoes into wild populations (as discussed in the final section of this review). The process of CI is explained below and summarized in Fig. 1.

Alterations in spermatogenesis during male development result in the failure of *Wolbachia*-infected males to produce offspring when mating with either uninfected females (unidirectional CI) or females infected with a different strain of *Wolbachia* (bidirectional CI) (Fig. 1). The altered sperm results in late entry of the male pronucleus into mitosis. Thus, the parental chromosomes fail to align properly and haploid embryos are produced. The presence of compatible *Wolbachia* in fertilized eggs, through vertical transmission, rescues CI (Landmann *et al.*, 2009; Stouthamer *et al.*, 1999; Tram *et al.*, 2006; Tram & Sullivan, 2002). Importantly, females infected with *Wolbachia* can mate successfully with uninfected males, giving them an evolutionary advantage over uninfected females, as they produce viable offspring with both infected and uninfected males (Turelli & Hoffmann, 1991, 1995; Yen & Barr, 1971).

As early as the 1960s, mosquitoes that exhibited CI were considered a viable option for the control of disease vectors. One study reported the large scale release of *Wolbachia*-infected *C. pipiens* into wild populations in order to eliminate this population from a large urban area (Laven, 1967). More recently, a virulent strain of *Wolbachia* found in laboratory strains of *D. melanogaster*, *w*MelPop, was cited as a possible mechanism for the control of mosquito populations and consequently the spread of arboviruses through transinfection (see below) (Brownstein *et al.*, 2003; Yeap *et al.*, 2011). This particular strain of *Wolbachia* is known to infect *Drosophila* at a high density and reduce lifespan. It has since been shown that it is possible to transinfect *Aedes albopictus* and *Ae. aegypti* populations with *w*MelPop and other *Wolbachia* strains, and for them to be stably maintained in populations (Blagrove *et al.*, 2013; McMeniman *et al.*, 2009; Xi *et al.*, 2005). The potential of *Wolbachia* to control arbovirus spread, however, was not fully realized until 2008 when two groups reported that *Wolbachia* infection in *D. melanogaster* led to increased protection against insect RNA viruses, suggesting that *Wolbachia* was able to control viral replication in infected flies (Hedges

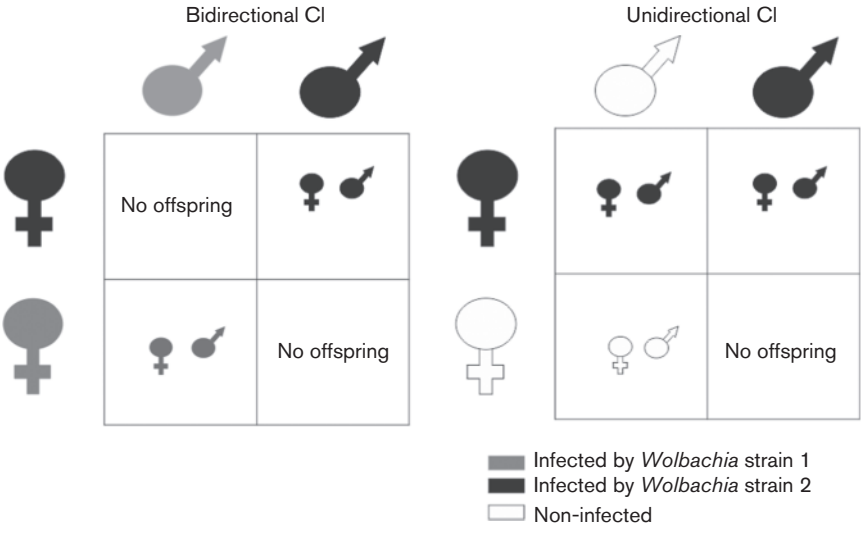

Fig. 1. CI induced by *Wolbachia* infection. CI is presented in two forms in insects. (a) The process by which bidirectional CI takes place. Here, males and females infected with two different strains of *Wolbachia* are unable to produce viable offspring. (b) The process of unidirectional CI, where infected females are able to successfully mate with both uninfected males and males infected with the same strain of *Wolbachia*.

et al., 2008; Teixeira *et al.*, 2008). The remainder of this review will discuss this in more detail.

Wolbachia and antiviral activity in insects

Studies in the model *Drosophila*

Initial studies carried out in *D. melanogaster* concluded that the *Wolbachia* strains *w*MelCS and *w*MelPop increased fly longevity upon infection with the RNA viruses *Drosophila* C virus (DCV; *Dicistroviridae*), Flock House virus (FHV; *Nodaviridae*) and cricket paralysis virus (CrPV; *Dicistroviridae*), but not the DNA virus insect iridescent virus 6 (IIV-6; *Iridoviridae*) (Hedges *et al.*, 2008; Teixeira *et al.*, 2008). Moreover, viral titres were significantly reduced in the presence of *Wolbachia* in the case of DCV and nora virus (NoraV; a potentially novel family within the *Picornavirales*).

Further studies in *Drosophila simulans*, which are naturally infected with multiple *Wolbachia* strains, showed that only those *Wolbachia* strains that are present in high densities (*w*Mel, *w*Ri and *w*Au), conferred antiviral resistance to DCV and FHV. In DCV-infected *D. simulans*, *Wolbachia*-mediated protection did not directly correlate with reduced viral copy numbers (Osborne *et al.*, 2009). For example, *w*Ri significantly increased lifespan upon infection with DCV compared to controls; however, there was no significant decrease in virus accumulation. Conversely, Teixeira *et al.* (2008) and Hedges *et al.* (2008) showed that DCV titres in *D. melanogaster* were strongly reduced by *Wolbachia* infection (Hedges *et al.*, 2008; Teixeira *et al.*, 2008). During FHV infection, virus titre was unaffected but virus-induced pathology was decreased, resulting in increased life spans of infected flies (Teixeira *et al.*, 2008). A recent study also demonstrated that replication of the dsRNA virus bluetongue virus (BTV; *Reoviridae*) in *D. melanogaster* is inhibited in the presence of *Wolbachia* (Shaw *et al.*, 2012). Taken together these data suggest that reduction of viral titres is not the only mechanism responsible for *Wolbachia*-mediated protection. These findings in the model organism *Drosophila* have led to considerable interest in the field of human-pathogenic mosquito-borne viruses. The key findings are summarized in Table 1.

Mosquitoes and *Wolbachia*-mediated antiviral activity

Aedes and *Culex* species are the major arthropod vectors for DENV, West Nile virus (WNV) and yellow fever virus (YFV) (all *Flaviviridae*), as well as CHIKV (*Togaviridae*) (Weaver & Reisen, 2010). Among the major arbovirus

Table 1. Antiviral effects of *Wolbachia* in different *Drosophila*/virus associations

Species	*Wolbachia* strain	Nature of host-*Wolbachia* association	Virus	Antiviral effect exerted by *Wolbachia*	Reference
Drosophila melanogaster	*w*MelCS	Natural	DCV	Increased lifespan of infected flies; reduced virus proliferation	(Teixeira *et al.*, 2008)
			FHV*	Increased lifespan of infected flies; no effect of virus replication	
			NoraV	Reduced virus proliferation	
			IIV-6	Decreased lifespan of infected flies; increased virus proliferation	
			DCV, FHV*, CrPV	Increased lifespan of infected flies; reduced virus proliferation	(Hedges *et al.*, 2008)
	*w*MelPop	Laboratory strain	DCV, FHV*, CrPV	Increased lifespan of infected flies; reduced virus proliferation	(Hedges *et al.*, 2008)
	*w*Mel	Natural	CHIKV*, LACV*	No effect	(Glaser & Meola, 2010)
			WNV*	Reduced virus proliferation	(Glaser & Meola, 2010)
			BTV*	Reduced virus proliferation	(Shaw *et al.*, 2012)
Drosophila simulans	*w*Mel	Transinfection	DCV	Increased lifespan of infected flies; reduced virus proliferation	(Osborne *et al.*, 2009)
	*w*Au	Natural	DCV	Increased lifespan of infected flies; reduced virus proliferation	(Osborne *et al.*, 2009)
			FHV*	Increased lifespan of infected flies;	(Osborne *et al.*, 2009)
	*w*Ri	Natural	DCV	Increased lifespan of infected flies	(Osborne *et al.*, 2009)
			FHV*	Increased lifespan of infected flies	(Osborne *et al.*, 2009)
	*w*Ha	Natural	DCV, FHV*	No effect	(Osborne *et al.*, 2009)
	*w*No	Natural	DCV, FHV*	No effect	(Osborne *et al.*, 2009)

BTV, Bluetongue virus; CHIKV, chikungunya virus; CrPV, cricket paralysis virus; DCV, *Drosophila* C virus; FHV, Flock House virus; IIV-6, insect iridescent virus 6; LACV, La Crosse virus; NoraV, nora virus; WNV, West Nile virus.

vectors, *Ae. albopictus*, *Aedes bromeliae* and members of the *C. pipiens* complex are naturally infected with *Wolbachia* endosymbionts (*Ae. albopictus* harbours *w*AlbA and *w*AlbB, and *C. pipiens w*Pip) (Armbruster *et al.*, 2003; Hertig & Wolbach, 1924; Kittayapong *et al.*, 2000; Osei-Poku *et al.*, 2012; Sinkins *et al.*, 1995), whereas *Ae. aegypti* mosquitoes lack this association (Table 2).

Unlike the above-mentioned native *Drosophila/Wolbachia* interactions, the ability of homologous *Wolbachia* strains to confer resistance in mosquitoes is limited by tissue density and distribution. For example, *w*AlbA and *w*AlbB did not inhibit DENV replication to significant levels in *Ae. albopictus* mosquitoes (Bian *et al.*, 2010), but reduced viral infection of the salivary glands and thus may limit transmission (Mousson *et al.*, 2012). Furthermore, the *w*AlbA and *w*AlbB infected *Ae. albopictus* cell line Aa23 showed decreased DENV titres compared to *Wolbachia*-cured controls. Notably this cell line has a significantly higher *Wolbachia* density than somatic tissues of *Ae. albopictus*, which may explain its restrictive phenotype (Lu *et al.*, 2012). A study of the interaction between CHIKV and *w*AlbA and *w*AlbB in *Ae. albopictus* mosquitoes showed no decrease in viral titres in the presence of *Wolbachia* compared to controls. Viral titres in *Wolbachia*-free mosquitoes were extremely variable compared to those harbouring *Wolbachia*, suggesting some level of interaction (Mousson *et al.*, 2010). Lastly, homologous *w*Pip infections in *Culex quinquefasciatus* resulted in reduced WNV titres and transmission rates (Glaser & Meola, 2010). Given that *Wolbachia* confers resistance to RNA viruses in *Drosophila* (Hedges *et al.*, 2008; Teixeira *et al.*, 2008), and that *Wolbachia* strains can be stably introduced and maintained in heterologous host mosquitoes (Blagrove *et al.*, 2013; McMeniman *et al.*, 2009; Xi *et al.*, 2005), multiple studies have investigated whether *Wolbachia* transinfection into different mosquito species would result in vectors refractory to infection with important viral pathogens.

Transinfection and resulting phenotypes

Transinfection of mosquitoes with heterologous *Wolbachia* strains is by no means trivial, as not all hosts are equally permissive and *Wolbachia* strains differ in their ability to stably transfect certain new host species (Werren *et al.*, 2008). Most likely as a consequence of evolutionary adaptation to their hosts, naturally occurring *Wolbachia* strains differ in their genome organization and in the reproductive phenotypes they cause (reviewed by Serbus *et al.*, 2008; Werren *et al.*, 2008). In nature, *Wolbachia* cross-species transmission events occur frequently (Haine *et al.*, 2005; Heath *et al.*, 1999; Huigens *et al.*, 2000, 2004; Kraaijeveld *et al.*, 2011; Vavre *et al.*, 1999; Werren *et al.*, 1995) and are thought to result in a loss of infection or, if infection is established, in a temporary increase in virulence, caused either by competition for resources or by disproportional host immune activation (Le Clec'h *et al.*, 2012; Lipsitch

Table 2. Antiviral effect of *Wolbachia* in different mosquito/arbovirus associations

Mosquito host	*Wolbachia* strain	Nature of host-*Wolbachia* association	Virus	Antiviral effect exerted by *Wolbachia*	Reference
Aedes aegypti	*w*AlbB	Transinfection	DENV	Reduced virus proliferation	Bian *et al.* (2010)
	*w*Mel	Transinfection	DENV	Blockage of viral proliferation	Walker *et al.* (2011)
			WNV	No effect	Hussain *et al.* (2013)
			CHIKV, YFV	Reduced virus proliferation	van den Hurk *et al.* (2012)
	*w*MelPop	Transinfection	DENV, CHIKV	Reduced virus proliferation	Moreira *et al.* (2009)
			YFV	Reduced virus proliferation	van den Hurk *et al.* (2012)
			WNV	Reduced virus proliferation	Hussain *et al.* (2013)
Aedes albopictus	*w*AlbA and *w*AlbB	Natural	CHIKV	No effect	Mousson *et al.* (2010)
			DENV	No effect on virus replication in midgut; reduced virus dissemination and transmission	
			DENV	No effect	Lu *et al.* (2012)
	*w*Mel (*w*AlbA and *w*AlbB present)	Transinfection	CHIKV	Reduced virus transmission	Blagrove *et al.* (2013)
Aedes polynesiensis	*w*PolA	Natural	DENV	No effect	Bian *et al.* (2013b)
	*w*AlbB	Transinfection	DENV	Reduced virus proliferation	Bian *et al.* (2013b)
Culex quinquefasciatus	*w*Pip	Natural	WNV	Reduced virus proliferation	Glaser & Meola (2010)
Armigeres subalbatus	Not subgrouped	Natural	JEV	No effect in salivary gland cells	Tsai *et al.* (2006)

CHIKV, Chikungunya virus; DENV, dengue virus; JEV, Japanese encephalitis virus; WNV, West Nile virus; YFV, yellow fever virus.

& Moxon, 1997). In order to stably introduce the *D. melanogaster Wolbachia* strain *w*MelPop into *Ae. aegypti*, McMeniman *et al.* (2008) first had to pre-adapt *w*MelPop to an *Ae. albopictus*-derived cell line by long-term serial passage (McMeniman *et al.*, 2008). After about 2.5 years in culture the pre-adapted strain was transferred into *Ae. aegypti* and *Anopheles gambiae* cell lines and passaged further. The resulting *Wolbachia* strain (named *w*MelPop-CLA) was then reintroduced into *D. melanogaster* cured of *w*MelPop, where it showed reduced virulence and ability to induce CI compared to *w*MelPop (McMeniman *et al.*, 2008). In *Ae. aegypti*, *w*MelPop-CLA induced life-shortening and CI (McMeniman *et al.*, 2009). Further to this, several species of *Wolbachia* from both *Drosophila* and mosquito species have been transinfected into different hosts, leading to different levels of antiviral activity (summarized in Table 2). For example, in *w*Mel-transinfected *Ae. aegypti*, WNV was not inhibited, whereas the presence of the *w*MelPop strain of *Wolbachia* significantly reduced WNV replication (Hussain *et al.*, 2013). Similarly, *w*MelPop-CLA, and to a lesser extent *w*Mel, was able to diminish YFV replication and dissemination in transinfected *Ae. aegypti* (van den Hurk *et al.*, 2012). In the same study it has been shown that *w*Mel can only inhibit infection and dissemination of CHIKV in transinfected *Ae. aegypti* upon viraemic bloodmeal, but not upon intrathoracic inoculation (van den Hurk *et al.*, 2012). More recently, *w*Mel transinfection in *Ae. albopictus* was found to prevent CHIKV dissemination to the salivary glands and thus possibly viral transmission (Blagrove *et al.*, 2013).

Further studies have shown that transinfection of *Ae. aegypti* with the *D. melanogaster w*MelPop-CLA strain of *Wolbachia* negatively affects mosquito survival and strongly inhibits the replication of DENV when compared to tetracycline-treated (cured of *Wolbachia* infection) control or wild-type mosquitoes, irrespective of whether mosquitoes were blood fed or whether virus was injected intrathoracically (Moreira *et al.*, 2009). *Wolbachia* also reduced DENV dissemination to the thorax and head of mosquitoes, and hence possibly transmission (Bian *et al.*, 2010; Moreira *et al.*, 2009). *Ae. aegypti* transinfected with the *Ae. albopictus Wolbachia* strain *w*AlbB also showed increased mosquito longevity upon infection with DENV and suppressed viral replication (Bian *et al.*, 2010).

It would appear that the *Wolbachia*-induced virus refractory phenotype is dependent on the combination of *Wolbachia* strain, virus and host (genetic or other) factors. Understanding these differences is key to a successful vector control protocol. Several mechanisms have been proposed to explain why *Wolbachia* inhibits arbovirus transmission effectively in some scenarios but not in others. These will be discussed below.

Determinants of *Wolbachia* viral interference in arthropod hosts

It is not known how *Wolbachia* endosymbionts confer antiviral resistance to their hosts, and in order to develop intervention strategies based on *Wolbachia*-mediated viral interference it is crucial to elucidate the underlying mechanisms. An important question to address is whether *Wolbachia* interferes with viral replication directly or whether it increases vector resistance to viral infection by other mechanisms. Furthermore, it is important to elucidate whether the *Wolbachia*-mediated effect is cell autonomous or systemic.

Wolbachia density and competition for host cell resources

Density. Early studies found a positive correlation between intracellular endosymbiont density and antiviral effect conferred (Lu *et al.*, 2012; Osborne *et al.*, 2009, 2012). Different *Wolbachia* strains are known to infect vector species at variable densities and to display different tissue distributions (Dobson *et al.*, 1999; Dutton & Sinkins, 2004; Miller & Riegler, 2006). In its native host *Ae. albopictus*, *w*AlbB is found both in reproductive tissues (ovaries, testes) and somatic tissues (haemolymph, midgut, muscle, head) (Dobson *et al.*, 1999). In somatic tissues, *w*AlbB levels can vary significantly between *Ae. albopictus* strains (Dobson *et al.*, 1999). In *C. pipiens*, *Wolbachia* densities can vary up to 100-fold between individual mosquitoes and also between populations, which could lead to differences in vector competence (Berticat *et al.*, 2002; Echaubard *et al.*, 2010; Glaser & Meola, 2010). In heterologous hosts, however, *Wolbachia* usually grows to higher densities than in native hosts, which may explain why antiviral effects are more often observed in heterologous hosts compared to native host species (Glaser & Meola, 2010). Examples of *Wolbachia* transinfections that resulted in increased bacterial densities and antiviral resistance compared to original vector species include infection of *Ae. aegypti* (Bian *et al.*, 2010) or *Aedes polynesiensis* (Bian *et al.*, 2013b) with *w*AlbB. An exception is *w*MelPop, which grows to high titres in *D. melanogaster* and leads to a reduced life span of the host and a reduction in viral replication (Hedges *et al.*, 2008; Min & Benzer, 1997).

It has been hypothesized that protection is dependent on whether *Wolbachia*-harbouring tissues and viral target cells or tissues co-localize (Bian *et al.*, 2010; Moreira *et al.*, 2009). Interestingly, in *C. pipiens Wolbachia* is less abundant in the midgut than in other somatic tissues (Dobson *et al.*, 1999) and may not be present to limit DENV replication in midgut epithelial cells (Glaser & Meola, 2010). Density may also be important if it is simply a case of competition for space and/or cellular resources. Indeed there is little evidence of *Wolbachia* and virus being present together in the same cell/tissue when either is at a high density (Frentiu *et al.*, 2010; Moreira *et al.*, 2009).

Autophagy. One mechanism that is thought to control the density of *Wolbachia* is autophagy. Infection of C6/36 cells (an *Ae. albopictus*-derived cell line) with *w*AlbB showed that *Wolbachia* not only induced the autophagy pathway

but also manipulated it in order to ensure survival (Voronin *et al.*, 2012). This was also true for a naturally infected *D. melanogaster* line (*w*MelPop) (Voronin *et al.*, 2012). Since both alphaviruses and flaviviruses are thought to require the autophagy pathway in order to replicate (Krejbich-Trotot *et al.*, 2011; Lee *et al.*, 2008), this cellular function may be a site of *Wolbachia* interference with virus replication. Further work is clearly required to confirm this proposal.

Iron metabolism and cholesterol. If the *Wolbachia*-mediated antiviral effect is cell autonomous, then one explanation for reduced virus replication may be competition for resources between *Wolbachia*, viruses and the host cell. For example, iron homeostasis has been shown to be regulated by *Wolbachia* (Kremer *et al.*, 2009), with transcriptomic data suggesting that iron-binding proteins, transferrin and ferritin, were upregulated in the presence of *w*Mel and *w*MelPop-CLA in *Ae. aegypti* (Rancès *et al.*, 2012). Data have further indicated that transferrin was downregulated upon infection with DENV and CHIKV (Tchankouo-Nguetcheu *et al.*, 2010).

In addition to iron homeostasis, the cholesterol metabolism needs to be tightly regulated to enable viral replication and bacterial growth. Insect cells are cholesterol auxotrophs (Clayton, 1964), and can be depleted of cholesterol and other lipids by growth in delipidated serum (Hafer *et al.*, 2009; Silberkang *et al.*, 1983); however, they will incorporate low levels of cholesterol from serum (Mitsuhashi *et al.*, 1983). *Wolbachia* is unable to synthesize cholesterol *de novo* and thus its replication is cholesterol-dependent (Lin & Rikihisa, 2003). Further, inside host cells individual bacteria are contained within cholesterol-containing Golgi-related vesicles (see also Fig. 2) (Cho *et al.*, 2011). Cholesterol depletion of host cells by *Wolbachia* could also interfere with virus replication. A recent study has examined the involvement of cholesterol in *Wolbachia*-mediated DCV interference in *D. melanogaster* (Caragata *et al.*, 2013). It was shown that cholesterol enrichment of fly diets reversed the *Wolbachia*-mediated DCV interference effect and the increase in *D. melanogaster* life span in a dose-dependent manner. Importantly, dietary cholesterol levels did not affect *Wolbachia* densities in *w*MelCS-infected flies and did not affect DCV infection levels in *Wolbachia*-free flies (Caragata *et al.*, 2013). For *w*MelPop-infected flies there was a significant effect of diet on *Wolbachia* density; however, this did not affect pathogen blocking (Caragata *et al.*, 2013).

Both mosquito-borne alphaviruses and flaviviruses have been shown to rely on host cell lipids for their uptake, replication, virion assembly and infectivity. Sindbis virus (SINV; *Togaviridae*) and Semliki Forest virus (SFV; *Togaviridae*) are endocytically taken up into host cells, followed by cholesterol and sphingolipid-dependent fusion of the viral and host membranes at low pH (Kielian, 1995; Lu *et al.*, 1999; Smit *et al.*, 1999). Their replication takes place in replication complexes on intracellular endosome-derived membranes. In insect cells, virion assembly takes place in cytoplasmic vesicles that fuse with the plasma membrane in a cholesterol-dependent manner to release virus into the medium (Brown & Condreay, 1986). Therefore, the lipid content of viral particles resembles that of the producer cells (Hafer *et al.*, 2009). Several studies have shown that alphaviruses can grow to high titres in insect cells although their lipid content is generally low (Hafer *et al.*, 2009; Mitsuhashi *et al.*, 1983; Tsetsarkin *et al.*, 2011). Cholesterol depletion of C6/36 cells blocked SFV endosome membrane fusion, replication and exit (Chatterjee *et al.*, 2000; Marquardt *et al.*, 1993; Phalen & Kielian, 1991). A significant reduction in the production of infectious SINV and SFV particles upon delipidation of C6/36 cells has been reported (Hafer *et al.*, 2009; Lu *et al.*, 1999; Marquardt *et al.*, 1993; Phalen & Kielian, 1991). It can be assumed that the lower infectivity of these particles was caused by an

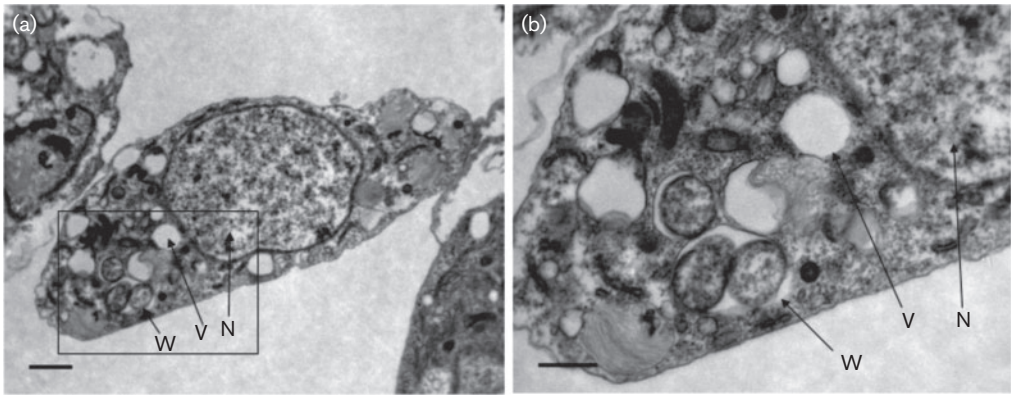

Fig. 2. Transmission electron micrographs of *Drosophila* JW18 cell line infected with *Wolbachia*. (a) Low magnification image of a whole cell depicting the distribution of *Wolbachia* (W) in *Drosophila* JW18 cells (Serbus *et al.*, 2012), with respect to the nucleus (N) and vacuoles (V). (b) Higher magnification of the boxed area of the cell in (a) showing a pair of *Wolbachia* cells in the same vacuole, presumably at the end of the process of division. Bars, 1 μm (a) and 0.5 μm (b).

altered lipid composition, which resulted in a loss of stability and structural fragility of the produced virions. Interestingly, in addition to cholesterol other lipids seem to be essential for virion infectivity (Hafer *et al.*, 2009).

Similar to alphaviruses, flavivirus replication relies on a tight regulation of host cell cholesterol biosynthesis (Carro & Damonte, 2013; Rothwell *et al.*, 2009). Although DENV was found to be insensitive to plasma membrane cholesterol depletion in C6/36 cells (Acosta *et al.*, 2008; Mosso *et al.*, 2008; Umashankar *et al.*, 2008), extraction of cholesterol from viral envelopes reduced DENV infectivity for C6/36 cells (Acosta *et al.*, 2009). The differential dependence on host cell membrane cholesterol content seen between members of the families *Togaviridae* and *Flaviviridae* may be explained by the fact that the former viruses bud through the host cell plasma membrane so that the lipid composition of their envelopes resembles that of the cell membrane (Hafer *et al.*, 2009; Sousa *et al.*, 2011). In contrast, the origin of the flavivirus envelope is the endoplasmic reticulum (Welsch *et al.*, 2009), which contains only a small proportion of the total cell cholesterol (Lange *et al.*, 1999). Given the crucial role for cholesterol and other lipids in viral life cycles, it is not surprising that another flavivirus, WNV, has been shown to modulate host cell cholesterol levels by upregulating cholesterol biosynthesis and redistributing it to sites of virus replication (Mackenzie *et al.*, 2007). These cellular modifications are necessary for WNV replication to take place. In summary, these early experiments relating to Wolbachia, cholesterol and antiviral activity are intriguing and warrant further investigation as it is clear that cholesterol is important both for virus replication and *Wolbachia* growth.

Immune signalling in arthropod/*Wolbachia* interactions

As *Wolbachia* is now known to confer resistance to a broad range of RNA viruses, it could be assumed that the mechanism(s) behind antiviral activity would also be broad ranging. Innate immunity consists of a set of anti-microbial mechanisms largely conserved between insect species and is the first line of defence against invading microbes, including viruses (Bartholomay *et al.*, 2010; Christophides *et al.*, 2002; Lemaitre & Hoffmann, 2007; Waterhouse *et al.*, 2007). It is therefore plausible to suggest that *Wolbachia* may control viral infection by pre-activating the immune system, also known as immune priming. Much of our understanding of innate immunity in mosquitoes is influenced by key findings made in *Drosophila* studies (Fragkoudis *et al.*, 2009; Lemaitre & Hoffmann, 2007; Merkling & van Rij, 2013; Wong *et al.*, 2011).

Toll, Imd and JAK-STAT

Among a number of mechanisms that limit bacterial infections, three major signalling pathways – Toll, Imd and JAK-STAT – control innate immunity. The Toll pathway is initiated upon infection with fungi or Gram-positive bacteria, leading to transcriptional upregulation of a specific subset of antimicrobial peptides (AMPs) and several other immune genes (Lemaitre & Hoffmann, 2007). The Toll pathway has also been implicated in antiviral immunity. For example, the pathway is activated by the presence of the dsRNA virus Drosophila X virus (DXV; *Birnaviridae*) in *Drosophila* (Zambon *et al.*, 2005), and has been implicated in the control of DENV and WNV in mosquito studies (Smartt *et al.*, 2009; Xi *et al.*, 2008). Stimulation with Gram-negative bacteria activates the Imd pathway, and induces immune genes and Imd-specific AMPs (Lemaitre & Hoffmann, 2007). Several mutants in the *Drosophila* Imd pathway also exhibit higher CrPV titres compared to wild-type flies (Costa *et al.*, 2009). Studies in *An. gambiae* suggest the differential expression of Imd pathway genes in response to o'nyong'nyong virus (ONNV; *Togaviridae*); however, it would only appear to have a minor role in that particular host–virus interaction (Waldock *et al.*, 2012). A third pathway known to be involved in both bacterial and viral infection is the JAK-STAT pathway. DCV, for example, activates the JAK-STAT pathway in *Drosophila* resulting in the upregulation of genes such as *vir1* (Dostert *et al.*, 2005). Studies in *Ae. aegypti* have demonstrated that DENV infection is controlled by JAK-STAT signalling (Souza-Neto *et al.*, 2009; Xi *et al.*, 2008). Indeed, knockdown of key components of the JAK-STAT pathway resulted in significantly higher DENV viral titres. In the case of alphaviruses, a number of studies in *Drosophila* and mosquito cells suggest that the combination of Imd and/or JAK-STAT signalling, but not the Toll pathway, has antiviral activities (Avadhanula *et al.*, 2009; Fragkoudis *et al.*, 2008; Huang *et al.*, 2013). Importantly, this shows that at least when it comes to antiviral immune signalling, differences between virus families, but also within virus families and vectors (SINV/SFV versus ONNV), exist.

Early studies in *Ae. aegypti* mosquitoes transinfected with several different strains of *Wolbachia* indicated that immune priming may be involved in antiviral activity. For example, the transinfection of *w*MelPop-CLA into *Ae. aegypti* resulted in the upregulation of immune genes, including several involved in the Toll and Imd pathways (Kambris *et al.*, 2010; Rancès *et al.*, 2012). Further transcriptional analysis of transinfected *Ae. aegypti* (*w*AlbB) showed significant upregulation of several Toll-dependent genes, in particular the AMP-encoding gene *defensin* (Bian *et al.*, 2010). These studies are, however, hampered by the initial transinfection of heterologous *Wolbachia* strains. Naturally infected *D. melanogaster*, *D. simulans*, and indeed, *Ae. albopictus* do not show this immune priming phenotype, and in the case of *D. simulans* and *D. melanogaster* (and to some extent *Ae. albopictus*) *Wolbachia* still confers resistance to several RNA viruses (Lu *et al.*, 2012; Osborne *et al.*, 2009; Rancès *et al.*, 2012; Teixeira *et al.*, 2008). Significantly, DENV infection is limited in both *D. melanogaster* and *Ae. aegypti*, which are infected with the strain *w*Mel, to similar degrees (Rancès *et al.*, 2012). Therefore, it is unlikely that the

immune priming seen in *Ae. aegypti* is key to *Wolbachia*-mediated protection, as there is no such priming in *D. melanogaster*. It has been shown that there was neither an induction nor suppression of AMPs in either *D. simulans* or *Ae. albopictus* naturally infected with different *Wolbachia* strains (see Tables 1 and 2, respectively) and these insects are furthermore able to mount a normal response to bacterial infection (Bourtzis *et al.*, 2000). Immune priming of the Imd or Toll pathways would also suggest that *Wolbachia* had the ability to protect against further bacterial infection; however, again this is not the case (Rottschaefer & Lazzaro, 2012). A more recent study has utilized the availability of *Drosophila* strains that are deficient in key components of the Imd and Toll pathway to conclude that neither a functional Toll nor an Imd pathway is required for the ability of *Wolbachia* to protect against DENV (Rancès *et al.*, 2013).

The Toll pathway has also been shown to be induced by the production of reactive oxygen species (ROS) in *Ae. aegypti* mosquitoes transinfected with the heterologous *Wolbachia* strain *w*AlbB (Pan *et al.*, 2012). The activation of the pathway in these mosquitoes leads to the production of the AMPs cecropin and defensin. Targeted reduction of these two AMPs resulted in an increase of DENV viral titres in these mosquitoes, leading to speculation that production of ROS and subsequent Toll activation may lead to *Wolbachia*-mediated antiviral activity. However, the corresponding experiment was not carried out in *Wolbachia*-free lines; therefore, the extent by which this activation leads to antiviral activity cannot be fully assessed (Pan *et al.*, 2012). The broad activity of *Wolbachia*-mediated protection would argue against immune priming as the main mediator of antiviral activity, given the different susceptibilities between viruses to Toll, Imd and JAK-STAT pathways.

A recent study identified the phenoloxidase (PO) cascade as an important mosquito innate immune response to SFV (Rodriguez-Andres *et al.*, 2012). Interestingly, *Wolbachia* has been shown to increase melanization through the PO cascade in both homologous and heterologous *Wolbachia*/host settings (Thomas *et al.*, 2011). It is likely that the PO cascade acts to protect against other viruses, and therefore the interaction with *Wolbachia* is intriguing and would merit further investigation.

Taken together, the studies described above suggest that immune priming is not central to the protection that *Wolbachia* confers. However, as transinfection of *Wolbachia* strains to heterologous hosts often leads to some level of immune priming, the additive effect of this cannot be ignored. It may be that the amount of antiviral protection present in released mosquitoes is vital to the sustainability of vector/arbovirus control. Studies suggest that as there is little or no immune priming present in homologous *Wolbachia*/host settings, over time co-evolution would lead to a reduction in the immune response. Therefore, it is crucial to understand to what extent immune priming plays a role in antiviral activity in transinfected hosts.

Small RNA pathways in arthropod/*Wolbachia* interactions

Further to the immune signalling pathways discussed above, mosquitoes respond to viral infection through the sequence-specific small RNA breakdown pathways, RNA interference (RNAi). RNAi is considered to be the most important antiviral response in insects and is key to the control of arboviruses in mosquitoes (Blair, 2011). The exact mechanisms behind antiviral RNAi are beyond the reach of this review (see Donald *et al.*, 2012 for a recent review). Nevertheless, as a mechanism that is known to successfully control a wide variety of arboviruses, it is important to ascertain if the RNAi response is involved in mediating the antiviral activity of *Wolbachia*. Several lines of evidence, however, suggest that it is not essential. Firstly, the *Ae. albopictus* cell line C6/36 does not contain a functional Dicer 2 protein, a key protein in the main antiviral RNAi response (Brackney *et al.*, 2010; Scott *et al.*, 2010). Transinfection of these cells with *w*Mel still results in the inhibition of DENV, suggesting that a functioning exogenous RNAi response is not necessary for inhibition to occur (Frentiu *et al.*, 2010). Additionally, *Drosophila* mutants defective for several key components of the antiviral RNAi pathway infected with *Wolbachia* still show resistance to WNV, DCV and FHV when compared to control flies (Glaser & Meola, 2010; Hedges *et al.*, 2012). Taken together these results indicate that the main antiviral RNAi pathway is not essential for *Wolbachia*-mediated viral protection.

In addition to the RNAi pathway, the microRNA (miRNA) pathway has been implicated in the pathogen-blocking effect of *Wolbachia* (Hussain *et al.*, 2011; Osei-Amo *et al.*, 2012; Zhang *et al.*, 2013). miRNAs are nonprotein-coding small RNAs that are involved in the regulation of cellular development, differentiation, apoptosis and immunity (Asgari, 2013; Donald *et al.*, 2012). Recently, the expression of miRNAs in *Ae. aegypti* mosquitoes transinfected with *w*MelPop-CLA was studied (Hussain *et al.*, 2011). The presence of *Wolbachia* has been shown to alter the expression of several insect miRNAs. The induction of one miRNA, aae-miR-2940, by *Wolbachia* is of particular interest to this review. aae-miR-2940 has been shown to downregulate the expression of the metalloprotease m41 ftsh (Hussain *et al.*, 2011). Inhibition of this miRNA or silencing of the metalloprotease gene in mosquitoes reduced *Wolbachia* density (Hussain *et al.*, 2011). Importantly, upregulation of aae-miR-2940 by *Wolbachia* has been found to inhibit DENV replication. This effect on DENV is mediated by the downregulation of the *Ae. aegypti* DNA (cytosine-5) methyltransferase gene *AaDnmt2*, a second target of aae-miR-2940 (Zhang *et al.*, 2013). DNA cytosine methylation is essential for host defence, genome stability, gene regulation, organ differentiation and ageing. Reversely, overexpression of *AaDnmt2* leads to a decrease in *Wolbachia* density, but an increase in DENV replication. In DENV-infected, *Wolbachia*-free mosquitoes, expression of *AaDnmt2* is upregulated. In *Wolbachia*-free *Ae. aegypti*, aae-miR-2940 is, although at low levels, expressed so that

AaDnmt2 levels are negligible (Zhang *et al.*, 2013). These findings point towards an important role of aae-miR-2940 in the maintenance of *Wolbachia* infection in *Ae. aegypti* as well as the resistance to DENV in *Wolbachia*-transinfected mosquitoes. Reduction in *AaDnmt2* expression in *Wolbachia*-infected cells could lead to hypomethylation of the mosquito genome and thereby regulate expression of methylation-sensitive host genes (Zhang *et al.*, 2013). In fact, a recent study has confirmed this idea (Ye *et al.*, 2013). In particular, expression of *Ae. aegypti* genes, with membrane functions, appear to be differentially regulated upon *Wolbachia* infection (Cho *et al.*, 2011; Ye *et al.*, 2013).

In summary, the studies discussed above suggest that the RNAi pathway is not essential for *Wolbachia*-mediated antiviral activity, but that the miRNA pathway could affect viral replication either directly, through up or down-regulation of genes involved in viral replication or antiviral activity, or indirectly, by altering the density of *Wolbachia*.

Applications, risks and future directions

Based on its pathogen-blocking effect *Wolbachia* introduction into wild mosquito populations has been suggested as a potential control measure for arboviruses. Previous introductions of genetically modified (GM) mosquitoes into wild populations have been met with public concern. However, *Wolbachia* is a naturally occurring symbiont of mosquitoes, and therefore infected vectors are not considered to be GM. Recent studies have also demonstrated that there is no antibody production against *Wolbachia* in human volunteers bitten by *Wolbachia*-infected mosquitoes, nor is there a transfer to the environment (be it mosquito predators, or soil, leaves, etc.) (Popovici *et al.*, 2010), suggesting that *Wolbachia* is a safe alternative to GM mosquitoes. Nevertheless, public engagement and detailed regulations, such as those set out by Hoffmann *et al.* (2011), are key to public and scientific acceptance of such release experiments.

Release of *Wolbachia*-infected mosquitoes into wild mosquito populations, with the aim of fixation of the infection frequency (i.e. stable introduction of *Wolbachia* in previously non-infected vector populations), requires extensive knowledge of local and regional mosquito population densities and dispersion dynamics (Engelstädter & Telschow, 2009; Schofield, 2002). Further parameters to consider include the strength of the *Wolbachia*-induced CI effect, maternal inheritance rates and fitness costs or advantages (for example refractoriness to viral infection) to the vector inferred by *Wolbachia* infection (Vavre & Charlat, 2012). Mathematical modelling approaches are then employed to calculate the minimum number of *Wolbachia*-infected mosquitoes to be released (Hancock & Godfray, 2012; Hoffmann *et al.*, 2011; Hughes & Britton, 2013; Turelli, 2010; Walker *et al.*, 2011).

A key question is how sustainable a *Wolbachia*-mediated approach would be. In order to answer this question, we firstly need to distinguish between two mechanisms by which stable introduction of *Wolbachia* into wild populations can limit vector competence for pathogen transmission: (i) *Wolbachia*-mediated life-shortening of mosquitoes, which would interfere with viral transmission, and (ii) *Wolbachia*-induced vector refractoriness to infection, which would limit viral replication and transmission (Vavre & Charlat, 2012). Modelling of the epidemiology of *Wolbachia* in host populations has suggested that vector/*Wolbachia* associations would be more sustainable in the field using the former approach as host resistance to the bacterium is less likely to occur (Read *et al.*, 2009; Schraiber *et al.*, 2012). However, it has been shown that mosquitoes carrying life-shortening *Wolbachia* strains, such as *w*MelPop, have to be released in very high numbers compared to mosquito populations present at the release site, and that they cannot invade regional mosquito populations (Hughes & Britton, 2013; McMeniman *et al.*, 2009; Walker *et al.*, 2011). In contrast to the reduction of viral transmission due to *Wolbachia*-induced life-shortening, vector refractoriness is expected to diminish over time as a function of vector/*Wolbachia* co-adaptation (Vavre & Charlat, 2012).

As discussed previously, the density of *Wolbachia* in transinfected mosquitoes, and indeed in homologous mosquito/*Wolbachia* combinations, is crucial to the *Wolbachia*-mediated antiviral activity (Lu *et al.*, 2012; Osborne *et al.*, 2009, 2012). Therefore, any decrease in *Wolbachia* density due to mosquito/*Wolbachia* co-adaptation may lead to a significant decrease in antiviral activity. However, it is clear from studies in *D. melanogaster* that long-term vector/*Wolbachia*/virus associations can lead to continued protection against viruses. For example, invasion of *D. melanogaster* by *w*Mel occurred during the last 80 years (Riegler *et al.*, 2005), yet *w*Mel still confers protection against DCV (Teixeira *et al.*, 2008). This is encouraging and further studies in this model system could be extremely beneficial to the field.

If, at some point, reintroductions of *Wolbachia*-infected mosquitoes are required, then understanding the mechanism of CI becomes important. CI will confer an evolutionary advantage to released mosquitoes over the wild population, which aids the spread and maintenance of *Wolbachia* within the wild population. It will, however, also limit the spread of newly introduced *Wolbachia* strains into the same population, as CI will prevent successful mating of the incoming mosquitoes with individuals of the original population (see Fig. 1).

Further to CI, the introduction of novel mosquito/*Wolbachia* combinations is hindered by the aforementioned technical difficulties of transinfections. As *Wolbachia* transinfection between closely related species is more successful (Bian *et al.*, 2010; Russell *et al.*, 2009), newly discovered *Wolbachia* strains of *Ae. bromeliae* (a YFV vector) and *Aedes metallicus* may be suitable candidates for introduction into aedine vectors (Osei-Poku *et al.*, 2012). Transinfection can also result in an evolutionary disadvantage within the mosquito, for example reduced fecundity

or viability. This in turn could affect the spread within a population. Coupling *Wolbachia* introduction to insecticide resistance has been suggested in order to counteract these effects (Hoffmann & Turelli, 2013). Spread of such resistance genes into wild populations requires careful evaluation and is dependent on local mosquito populations and control strategies.

The impact of introduced *Wolbachia* on the target virus may be complex. Modelling indicates that different *Wolbachia* strains have varying abilities to reduce transmission frequencies (R_0) (Bian *et al.*, 2010; Hoffmann *et al.*, 2011; McMeniman *et al.*, 2009), and if transmission is only partially blocked *Wolbachia* will place the target virus in a bottleneck, perhaps leading to the evolution of a virus resistant to *Wolbachia*-mediated protection. Furthermore, modelling of the co-evolution of parasites and protecting symbionts suggests increases in parasite virulence (Jones *et al.*, 2011). The implication – that exposure to *Wolbachia* might result in increased virulence of DENV or CHIKV – is of obvious concern. There is clearly a need for long-term studies, perhaps in tissue culture, of the impact of *Wolbachia* on virus evolution.

Despite these complications, initial trials with transinfected mosquitoes are encouraging. Following cage experiments with *Ae. aegypti* and *w*AlbB (Xi *et al.*, 2005), experiments with *w*Mel and caged *Ae. aegypti* in semi-field conditions have shown rapid establishment of infection in the insect population and inhibition of DENV replication (Walker *et al.*, 2011). Release experiments into the Australian environment have achieved spread of *w*Mel into two natural *Ae. aegypti* populations within months of release of infected adult mosquitoes (Hoffmann *et al.*, 2011), with no detectable adverse effects on the environment and human health. Indeed, new field trials are set to begin in Australia (http://www.eliminatedengue.com/progress).

Wolbachia offers a unique opportunity to potentially understand and control arbovirus transmission. Indeed, more recent studies have indicated the use of *Wolbachia* to control *Plasmodium* infections, opening the field further (Bian *et al.*, 2013a). For virologists, investigating and potentially manipulating the mechanisms underlying the inhibition of virus transmission by *Wolbachia* will remain an interesting and challenging task.

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