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(54) Title: MOSQUITO-ADAPTED BACTERIUM AGAINST HIGHLY INFECTIOUS MOSQUITO-BORNE VIRUSES

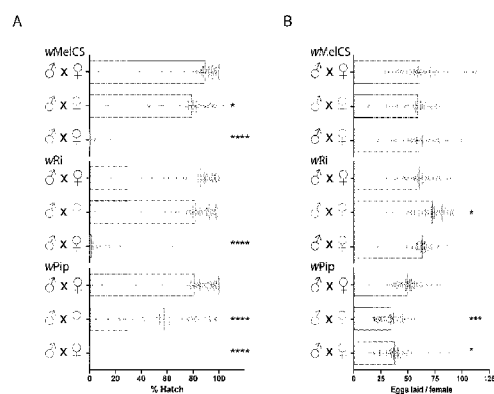


Figure 1

(57) Abstract: A mosquito-adapted *Wolbachia pipientis* var *wMelCS* bacterium is provided which is particularly efficacious for infecting mosquitoes that can harbour highly infectious viruses. Mosquitoes infected with the *wMelCS* bacterium may be released into the environment thereby preventing, reducing or eliminating a disease caused by, or associated with, the highly infectious mosquito-borne virus.



TITLEMOSQUITO-ADAPTED BACTERIUM AGAINST HIGHLY INFECTIOUS
MOSQUITO-BORNE VIRUSESFIELD OF THE INVENTION

5 THIS INVENTION relates to environmental control of mosquito-transmitted diseases. More particularly, this invention relates to a *Wolbachia* strain that is particularly useful in modifying mosquitoes that harbour highly infectious, disease causing viruses.

BACKGROUND OF THE INVENTION

10 Arboviruses transmitted by the *Aedes aegypti* mosquito, including dengue (DENV), Zika (ZIKV) and chikungunya (CHIKV) are emerging threats that impose an increasing health burden on tropical and subtropical regions of the world. While these viruses usually cause self-limiting febrile disease, severe manifestations such as hemorrhagic shock can lead to death. In the absence of
15 specific antiviral therapeutics and suboptimal vaccines (1-4), treatment is supportive only and limiting virus transmission has been largely dependent on vector control. The increasing global incidence of these diseases demonstrates the lack of effectiveness of current control programs and the need for novel, efficacious and cost-effective alternatives (5).

20 In 2011, the first releases of *Ae. aegypti* mosquitoes carrying the endosymbiotic bacterium *Wolbachia pipientis* began in field trials in Northern Australia as part of the Eliminate Dengue Program. *Wolbachia* is a gram negative, obligate endosymbiont that is maternally transmitted and can impart antiviral properties to arthropod hosts. It is estimated that at least 40% of all terrestrial
25 arthropod species are infected with *Wolbachia* (6) which can also manipulate host biology to induce feminization, parthenogenesis, cytoplasmic incompatibility (CI) and male-killing (7, 8). Of these traits, CI is probably the most common, driven by many *Wolbachia* strains, and enables the spread of *Wolbachia* into a host population by providing females with *Wolbachia* an indirect reproductive
30 advantage. In crosses between uninfected females and *Wolbachia*-infected males, CI results in the embryonic lethality of offspring. However, *Wolbachia*-infected

females can mate with both infected and uninfected males successfully. CI, coupled with the maternal transmission of *Wolbachia* leads to the rapid invasion of the host population (7, 8).

5 The antiviral activity *Wolbachia* can provide to its host is a more recently described phenomenon. The mechanism that drives this protection is not well understood but may involve priming of the insect innate immune response pathways in new *Wolbachia* infections, and competition for resources such as cholesterol (9-11). Field trials to date have utilized *Ae. aegypti* transinfected with the *Drosophila melanogaster* native *Wolbachia* strain, wMel, and a more
10 pathogenic form, wMelPop-CLA, isolated from a mutant lab strain of *D. melanogaster* (12-14). While these strains have been shown to restrict replication and dissemination of several virus genera including flaviviruses and alphaviruses in *Ae. aegypti*, due to the technical difficulties of introducing *Wolbachia* into a new species, the full potential of using alternative *Wolbachia* strains has remained
15 largely unexplored.

SUMMARY OF THE INVENTION

The present invention relates to the use of a mosquito-adapted *Wolbachia* strain wMelCS bacterium to modify mosquitoes for release into areas where high titre, disease-causing viruses are endemic, to thereby at least partly prevent or
20 inhibit viral disease transmission by mosquitoes.

In a broad form, the invention relates to a mosquito-adapted wMelCS bacterium for use in modifying mosquitoes capable of transmitting highly infectious viruses.

In an embodiment, the wMelCS bacterium is *Wolbachia pipientis* var wMelCS deposited at the National Measurement Institute, 1/153 Bertie St. Port
25 Melbourne, Victoria, 3207, Australia under accession number V17/025608 on 14 November 2017.

In a first aspect, the invention provides a mosquito infected with a mosquito-adapted wMelCS bacterium and optionally infected with a highly
30 infectious virus.

In a second aspect, the invention provides a population of mosquitoes that include one or more mosquitoes infected with a highly infectious virus and one or more mosquitoes that comprise *wMelCS* bacteria.

In a third aspect, the invention provides a method of modifying an environment that comprises mosquitoes harbouring a highly infectious virus capable of being transmitted by one or more of the mosquitoes in the environment, including the step of introducing one or more mosquitoes that comprise *wMelCS* bacteria into the environment to thereby modify the environment.

In a fourth aspect, the invention provides a method of modifying a mosquito population, including the step of introducing one or more mosquitoes that comprise *wMelCS* bacteria into the mosquito population to thereby modify the mosquito population, wherein the mosquito population comprises mosquitoes that harbour a highly infectious virus capable of being transmitted by one or more mosquitoes.

In a fifth aspect, the invention provides a method of at least partly suppressing or inhibiting transmission of a highly infectious virus within a vertebrate host population having one or more vertebrate host individuals harbouring the highly infectious virus, including the step of introducing one or more mosquitoes that comprise *wMelCS* bacteria into an environment comprising the vertebrate host population to thereby at least partly suppress or inhibit transmission of the highly infectious virus within the vertebrate host population.

In a sixth aspect the invention provides of at least partly preventing, reducing or eliminating a disease caused by, or associated with, a highly infectious mosquito-borne virus, said method including the step of introducing one or more mosquitoes that comprise *wMelCS* bacteria into an environment where the highly infectious mosquito-borne virus is, or may become, present to thereby at least partly prevent, reduce or eliminate the incidence of the disease in said environment.

In a seventh aspect, the invention provides a mosquito-adapted bacterium which is *Wolbachia pipientis* var *wMelCS* deposited at the National Measurement

Institute, Port Melbourne, Victoria, Australia under accession number V17/025608 on 14 November 2017.

In one embodiment of the aforementioned aspects, said mosquito is of the genus *Aedes*.

5 In a preferred embodiment, said mosquito is of the species *Aedes aegypti*.

Non-limiting examples of viruses include dengue virus, Zika virus and chikungunya virus, inclusive of all strains and serotypes of these viruses.

Suitably, the *wMelCS*-infected mosquitoes display substantially repressed or inhibited virus replication, substantially normal maternal transmission and/or
10 substantially normal cytoplasmic incompatibility (CI), preferably at minimal fitness cost to the mosquito.

Throughout this specification, unless otherwise indicated, “comprise”, “comprises” and “comprising” are used inclusively rather than exclusively, so that a stated integer or group of integers may include one or more other non-stated
15 integers or groups of integers.

It will also be appreciated that the indefinite articles “a” and “an” are not to be read as singular indefinite articles or as otherwise excluding more than one or more than a single subject to which the indefinite article refers. For example, “a” mosquito includes one mosquito, one or more mosquitoes or a plurality of
20 mosquitoes.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1. CI, fecundity and egg diapause viability in *wMelCS*, *wRi*, and *wPip* transinfected *Ae. aegypti*. (A) Hatch rates for crosses between infected and uninfected mosquitoes show CI and successful hatching. Bars are the mean
25 percentage of eggs hatched +/- SEM from >40 females (individual data points are superimposed). Symbols for tetracycline-treated mosquitoes (uninfected) are in black, *Wolbachia*-infected are in red. Asterisks indicate significance compared to Tet x Tet controls (Kruskal-Wallis, Dunn's, * for <0.05, *** for <0.001, **** for <0.0001). (B) Fecundity was determined as a measure of eggs laid per female.
30 Symbol codes and statistics are as per (A). Data are the mean +/- SEM from >40 females (individual data points are superimposed). (C) Eggs from gravid females

were collected over 72 h, from 3-days post blood meal. Eggs were dried slowly over 3-5 days then stored in a humid, airtight container. Batches of 100-500 eggs were hatched after 1, 2, 3, 4, 6, 8, 10 and 12 weeks. Hatched larvae were counted at 2nd instar stage until no hatch was observed for a week then, the percent hatch

5 calculated. Statistical analysis was performed using 2way ANOVA Sidak's multiple comparisons test. *wPip* hatch rate was significantly reduced at all weeks compared to *wPip.Tet* ($p < 0.0001$). *wMel* hatch rate was significantly reduced at weeks 4, 8, 10, ($p < 0.05$) and 12 ($p < 0.0001$) compared to *wMel.Tet*. *wMelCS* had a significantly reduced hatch rate at weeks 10 and 12 compared to *wMelCS.Tet*

10 ($p < 0.01$ and $p < 0.0001$, respectively). *wRi* had a significantly reduced hatch rate at week 12 only, compared to *wRi.Tet* ($p < 0.0001$).

Figure 2. Longevity in transinfected *Ae. aegypti* lines

Age-controlled (emergence within 24 h) adults (~150 males and ~150 females) were maintained at 26°C, 65% relative humidity and a 12:12 h light:dark cycle in

15 a climate controlled room. The number of dead males and/or females was recorded and carcasses removed daily until all mosquitoes in the cages were dead. Significant differences were observed for *wRi* females ($p < 0.0001$), *wMel* ($p < 0.05$) and *wMelCS* ($p < 0.05$) males, relative to their respective Tet control line. *wPip* had a significantly shorter lifespan for both males and females compared its

20 matched Tet-control ($p < 0.0001$). Statistical analysis was performed using a Log-rank (Mantel-Cox) test.

Figure 3. *Wolbachia* density and distribution in transinfected *Ae. aegypti* lines

(A) Density of *Wolbachia* within 5-day old whole female mosquitoes was determined by qPCR using primers directed to the conserved *16S* rRNA gene.

25 Data are the mean and SEM of 24 mosquitoes (individual data points are superimposed). Asterisks indicate significance compared to *wMel* (Mann-Whitney test, **** $p < 0.0001$). (B) The distribution of *wMelCS*, *wRi* and *wPip* *Wolbachia* strains in mosquitoes was determined in sections of paraffin-embedded female mosquitoes (5 to 7 day old) using fluorescence *in situ*

30 hybridisation (FISH). The fluorescently labelled 16S probe detects the *16S* rRNA gene from all four *Wolbachia* strains. Total DNA was stained in blue using DAPI

and a green filter was included to increase contrast with surrounding tissues. *Sg* indicates salivary gland tissue, *m* indicates muscle, and *c* indicates cardia. White arrows identify select regions of *Wolbachia* staining.

Figure 4. *wMelCS* and *wRi* *Wolbachia* strains inhibit DENV-3 replication and dissemination following an infectious blood meal.

Seven-day old female mosquitoes were fed a blood meal of fresh DENV-3 (2.0×10^6 TCID₅₀/ml) mixed 1:1 with sheep blood ($n = 500$ per *Wolbachia*-infected line, $n = 200$ per respective Tet control line). Mosquitoes were sorted immediately to identify those that took a blood meal, and incubated for 14 days. Mosquito heads were then separated from the thorax and total RNA was extracted from each head and remaining mosquito body. DENV genome copies were determined by qRT-PCR for each body as a measure of infection, and for each head as a measure of viral dissemination. Data are the mean genome copies per mosquito body (top) or head (bottom) $\pm$ SEM, and are representative of 3 independent experiments. Statistical analyses were performed using a Mann-Whitney test where ** $p < 0.01$, **** $p < 0.0001$.

Figure 5. *wMelCS* provides superior blocking of DENV-3 genome replication in a mosquito injection challenge model.

(A) DENV-3 was injected into the thorax of 6 or 7-day old female mosquitoes at 2.5×10^6 TCID₅₀/ml (undiluted) or 10, 100 or 1000-fold dilutions thereof. RNA was extracted from whole mosquito bodies 7-days post infection and virus replication was quantified by qRT-PCR. Data are the mean number of genome copies per mosquito $\pm$ SEM. Number of DENV-3 positive mosquitoes/total n are indicated above each bar. ** $p < 0.01$, **** $p < 0.0001$, Mann-Whitney test. (B) The mean DENV genome copies and SEM from (A) are replotted as a function of virus concentration injected. Significant differences in the mean RNA copies of *wMel* and *wMelCS* lines are indicated by ** ($p < 0.01$). Significant differences in the mean RNA copies of *wMel.Tet* and *wMelCS.Tet* lines are indicated by ‡ ($p < 0.05$), Mann-Whitney test.

DETAILED DESCRIPTION

The present invention is predicated on the surprising discovery that mosquito adaptation of the *Drosophila melanogaster*-infecting bacterium *wMel*

CS results in a mosquito-adapted bacterium that is particularly useful for infecting mosquitoes to at least partly reduce or eliminate transmission of highly infectious viruses carried by mosquitoes. This occurs with no substantial fitness cost or loss of cytoplasmic incompatibility (CI). Thus, it is expected that the mosquito-adapted wMelCS strain may prove to have higher viral blocking characteristics in the field than other commonly used strains, such as wMel and wMelPop-CLA, without the fitness costs and loss of CI that prevent field establishment of some strains.

In an embodiment, the mosquito-adapted wMelCS strain bacterium is *Wolbachia pipientis* var wMelCS deposited at the National Measurement Institute, Port Melbourne, Victoria, Australia under accession number V17/025608 on 14 November 2017.

For the purposes of this invention, by “*isolated*” is meant material that has been removed from its natural state or otherwise been subjected to human manipulation. Isolated material may be substantially or essentially free from components that normally accompany it in its natural state, or may be manipulated so as to be in an artificial state together with components that normally accompany it in its natural state.

As used herein “*mosquito*” and “*mosquitoes*” include insects of the family *Culicidae*. Preferably, mosquitoes are of the sub-families *Anophelinae* and *Culicinae*. Even more preferably, mosquitoes are capable of transmitting disease-causing pathogens, including viruses, protozoa, worms (*e.g.* nematodes) and bacteria to a mammalian host. Non-limiting examples include species of the genus *Anopheles* which transmit malaria pathogens, species of the genus *Culex*, and species of the genus *Aedes* (*e.g.* *Aedes aegypti*, *Aedes albopictus* and *Aedes polynesiensis*) which transmit nematode worm pathogens, arbovirus pathogens such as *Alphaviruses* (*e.g.* Eastern Equine encephalitis, Western Equine encephalitis, Venezuelan equine encephalitis and Chikungunya virus), *Flavivirus* pathogens that cause diseases such as Japanese encephalitis, Murray Valley Encephalitis, West Nile fever, Yellow fever, Dengue fever and Zika virus-associated conditions such as Zika fever, microencephaly and Guillain-Barre

syndrome, and *Bunyavirus* pathogens that cause diseases such as LaCrosse encephalitis, Rift Valley Fever, and Colorado tick fever, although without limitation thereto.

Non-limiting examples of pathogens that may be transmitted by *Aedes aegypti* are dengue virus, Zika virus, yellow fever virus, chikungunya virus and heartworm (*Dirofilaria immitis*).

Examples of pathogens that may be transmitted by *Aedes albopictus* include West Nile Virus, yellow fever virus, St Louis Encephalitis virus, dengue virus, Zika virus and chikungunya virus although without limitation thereto.

In one embodiment, said mosquito is of the genus *Aedes*.

In a preferred embodiment, said mosquito is of the species *Aedes aegypti*.

Non-limiting examples of virus pathogens include dengue virus, Zika virus and chikungunya virus, inclusive of all strains and serotypes of these viruses.

In certain aspects, the invention relates to a mosquito-adapted wMelCS strain bacterium and/or its use to infect mosquitoes that are capable of harbouring and/or transmitting highly infectious viruses. By “mosquito-adapted” bacterium is meant a bacterium that has been taken out of its native host environment and adapted to a mosquito host, in which environment said bacterium does not naturally reside. Accordingly, wMelCS is a mosquito-adapted *Wolbachia pipientis* bacterium that has been isolated from its native host (*i.e.* *Drosophila melanogaster*) and adapted to infect, colonize or reside in a mosquito.

Particular aspects of the invention provide methods whereby wMelCS-infected mosquitoes are introduced into an environment that has, or could have, a population of wild mosquitoes that are infected with a highly infectious virus such as hereinbefore described. The wMelCS-infected mosquitoes may be particularly efficient with regard to reducing or eliminating virus transmission by the mosquitoes to a vertebrate host population in that environment. This may result in at least partial reduction or elimination of the incidence of the viral disease in the host population.

A “*vertebrate host*” may be any vertebrate animal upon which a mosquito feeds and/or to which a mosquito is capable of transmitting a disease-causing pathogen. Non-limiting examples of vertebrate hosts are mammals such as humans, domesticated pets (*e.g.* dogs and cats), wild animals (*e.g.* monkeys, rodents and wild cats) livestock animals (*e.g.* sheep, pigs, cattle, and horses) and avians such as poultry (*e.g.* chickens, turkeys and ducks), although without limitation thereto.

Suitably, the disease is caused by, or associated with, a virus pathogen which is a “highly infectious” virus. By “highly infectious” is meant a virus which is present at, or is capable of being present at, a load, titre, concentration or amount in a vertebrate host (*e.g.* following infection and/or replication) to the extent that the transmission rate of virus to wild-type (*i.e.* not modified *wMelCS*) mosquitoes feeding on the vertebrate host is at least 30%, 40%, 50%, 60%, 70% or more. The viral titre or load that relates to an at least 30% transmission rate to mosquitoes may vary between different viruses and/or between different isolates or serotypes of the same virus. By way of example in relation to dengue virus, viral loads or titres in human plasma for DENV-1 and DENV-2 that can result in a 50% transmission rate are typically 6.29–6.51 log₁₀ viral RNA copies/mL, whereas for DENV-3 and DENV-4 the load or titre is 7.49–7.52 log₁₀ viral RNA copies/mL (Carrington & Simmons, 2014, *Front. Immunol.* doi.org/10.3389/fimmu.2014.00290).

Although not wishing to be bound by theory, it is proposed that a dose–response relationship exists such that increasing viral load or titre in a vertebrate host correlates with an increased likelihood of a mosquito becoming infected following feeding on the vertebrate host, up to the point of saturation. Thus, *wMelCS*-modified mosquitoes feeding on a vertebrate host infected with a highly infectious virus will inhibit or suppress viral replication more so than a wild-type mosquito (*i.e.* not modified by *wMelCS*), thereby inhibiting or suppressing viral transmission to other vertebrate hosts.

Suitably, the *wMelCS* bacteria do not produce or elicit a substantial fitness cost to *wMelCS*-infected mosquitoes. Examples of fitness costs include impaired

egg viability and larval development, although without limitation thereto.

Suitably, the *wMelCS*-infected mosquitoes display substantially repressed or inhibited virus replication, near-complete maternal transmission and substantially normal cytoplasmic incompatibility (CI) to enable rapid spread into a wild mosquito population, whilst inducing minimal fitness cost to the mosquito host.

Suitably, release of the *wMelCS*-infected mosquitoes into the environment modifies one or more other biological properties of said mosquito population. The one or more biological properties may include fecundity, tolerance of eggs to desiccation, pathogen susceptibility, viability of offspring and/or average life-span of a mosquito or mosquito population. A more detailed discussion of these biological properties in the context of *Wolbachia*-infected mosquitoes may be found in United States Patent 9090911, for example.

So that the invention may be fully understood and put into practical effect, the skilled reader is directed to the following non-limiting detailed Examples.

EXAMPLES

INTRODUCTION

This study set out to examine whether we could improve on the pathogenic protection afforded to *Ae. aegypti* by *wMel*, whilst avoiding fitness costs to the mosquito such as impaired egg viability and larval development as described for *wMelPop-CLA* infections (15-18).

For a *Wolbachia*-transinfected *Ae. aegypti* line to be considered for future release, the *Wolbachia* strain must provide strong protection against virus replication, demonstrate near-complete maternal transmission, induce CI to enable rapid spread into the wild mosquito population, whilst inducing minimal fitness cost to the mosquito host.

To predict which *Wolbachia* strains may provide these traits we looked to past studies, most of which have been performed using natively *Wolbachia*-infected, artificially transinfected, or introgressed *Drosophila* lines, with the RNA viruses *Drosophila C virus* (DCV) or *Flock House virus* (FHV) as models. From these studies, we identified *wMelCS* (from *D. melanogaster*) and *wRi* (from *D.*

simulans) as key candidates (19-22).

Although *Ae. aegypti* mosquitoes do not naturally carry *Wolbachia*, a number of mosquito species have natural infections including the closely related species *Aedes albopictus* (wAlbA and wAlbB) and *Aedes notoscriptus* (wNoto) as well as more distantly related species such as *Culex quinquefasciatus* (wPip). wAlbB has previously been transinfected into *Ae. aegypti* and shown to provide strong protection against DENV while having limited effects on host fitness (11, 23-25), suggesting that *Wolbachia* strains from more-closely related species could be better adapted for an *Ae. aegypti* host. wPip has been reported to provide protection against West Nile virus (WNV) in its natural host, identifying this strain as a possible candidate for transinfection in *Ae. aegypti* (26).

MATERIALS AND METHODS

Mosquito rearing

All *Ae. aegypti* mosquitoes were reared and maintained as described earlier (15, 24). Adult mosquitoes were maintained at 26°C, 65% relative humidity (RH) and a 12:12 h light:dark cycle in a climate-controlled room. Mosquitoes were blood fed on the arms of human volunteers (Monash University human ethics permit CF11/0766-2011000387). The *Wolbachia*-infected wMel line included in our experiments has been described previously (14, 15, 27). We generated uninfected lines for each strain by treatment of the infected lines with the antibiotic tetracycline (Tet) as described earlier (15, 28).

To exclude any influence of age and quality of eggs used in experiments, 5-day old females from all lines were fed by the same volunteer in one sitting. The eggs collected from these females were then dried and used as experimental material within 2-3 weeks. For experiments using the wPip line, freshly laid eggs were used due to poor hatch rates following egg storage.

To exclude any influence of mosquito age on our experiments, age-controlled adults emerging within a 24 h window were used (29).

Wolbachia transinfection

The *Wolbachia* uninfected and inbred PGYP1.Tet line was used as the recipient line for transinfection. Embryonic microinjection, isofemale line establishment and selection for stably-infected lines were done as previously described (14, 15) with a few modifications. In brief, the *wMelCS*, *wRi* and *wPip* strains were purified from the donor *wMelCS*-infected *Drosophila melanogaster*, *wRi*-infected *Drosophila simulans* and *wPip*-infected *Culex quinquefasciatus*, respectively, and microinjected into the posterior-pole of pre-blastoderm embryos of the recipient PGYP1.tet using methodology previously described (14, 15). Surviving G0 adult females from microinjection were mated to PGYP1.tet males, blood fed and setup for oviposition as isofemales. G0 females that laid fertile eggs were screened using quantitative PCR (qPCR) as described below. Once the newly introduced *Wolbachia* strain was at 100% frequency in the population and maintained itself for at least 2 consecutive generations the line was considered stable. The *wMelCS* and *wRi* lines were stable at G3 and *wPip* at G5.

Cytoplasmic incompatibility and maternal transmission

To investigate the level of *Wolbachia*-induced cytoplasmic incompatibility (CI) we set up paired crosses between *Wolbachia*-infected and tetracycline-treated (Tet) *Ae. aegypti*. For each *Wolbachia* strain four crosses were set up: (1) a CI cross (Tet females x infected males), (2) a maternal transmission (MT) cross (infected females x Tet males), (3) an uninfected control cross (Tet females x Tet males) and (4) an infected control cross (infected females x infected males). Each cross consisted of a group of 70 virgin males and 70 virgin females and each group was allowed to mate for 5 d after emergence. On day five all females were blood fed by one human volunteer. Gravid females were aspirated into individual tubes three-days post blood feeding and allowed to oviposit on wet filter paper (24). Three-days post oviposition female mosquitoes were removed and tested for the presence of *Wolbachia* by qPCR using strain-specific primers as described in the PCR section.

Any paper containing less than 15 eggs was discarded. The egg papers from the remaining females were photographed and eggs were counted manually.

The papers were then submerged in hatching water. Two days later the number of hatched larvae (2nd instar) was counted. Newly hatched larvae were counted daily until no hatching was recorded for three consecutive days. Any unhatched eggs were dried briefly for 48-72 h, hatched and counted again to get the maximum hatch.

To quantify the success of MT of *Wolbachia* to the next generation, hatched larvae from the MT cross (infected females x Tet males) were reared to 5-day old adults and screened by qPCR for the presence of *Wolbachia*.

10 ***Fitness determinants***

Egg diapause viability. To assess the viability of eggs stored over time, age-controlled adults (emergence within 24 h; 100 males and 100 females) were placed in quadruplet in 30x20x20cm cages. One cage of each line was blood-fed by the same human volunteer at 5 days post emergence. To control for the potential variation caused by blood-meal quality and composition. This was repeated 4 times with 4 different volunteers. Three-days post blood meal, 12 oviposition cups were placed in each cage for three days. Approximately 72 h post oviposition, egg cups were removed, dried slowly over 3-5 days then stored in Whirlpak bags (Sigma) in an airtight container with saturated KCl solution to maintain humidity (15). Batches of 100-500 eggs were photographed and counted, then hatched after 1, 2, 3, 4, 6, 8, 10 and 12 weeks from each cage. Hatched larvae were counted at 2nd instar stage until no hatch was observed for a week then the percent hatch calculated.

25 *Fecundity and hatch rate.* Performed using the parameters described for cytoplasmic incompatibility and maternal transmission above. Fecundity was calculated as the average number of eggs laid/female, and hatch rate determined as the number of 2nd instar larvae per 100 eggs hatched.

30 *Adult longevity.* Age-controlled adults (approximately 150 males and 150 females) were placed in triplicate 30x30x30cm cages. Mosquitoes were provided

fresh 10% sucrose twice/week and maintained at 26°C, 65% RH and a 12:12 h light:dark cycle in a climate-controlled room. The number of dead males and/or females was recorded and removed daily until all mosquitoes in the cages were dead (15, 24).

5

Wolbachia detection by PCR

The presence or absence of *Wolbachia* in transinfected mosquitoes was confirmed by qPCR using strain-specific primers. *wPip* was detected by amplifying an IS2 transposon, with 49 identical copies of this sequence in the *wPip* genome (forward primer (SEQ ID NO:1): 5'-GCACTTACCCTAACCAAAGGTAAC-3', reverse primer (SEQ ID NO:2): 5'-CTAACTTTAGGCCTCTATCGAAGAG-3'), *wRi* was detected by amplifying a part of the gene *Wri_009390*, which encodes a hypothetical protein (forward primer (SEQ ID NO:3): 5'-CATGCCAATAACGAAATAGC -3', reverse primer (SEQ ID NO:4): 5'-TAGCAACTTTTCTTGCGAAC-3'). A combination of two primer sets was used to differentiate *wMelCS* from *wMel* and *wMelPop-CLA* strains. One of the primers sets binds to *wMel* and *wMelCS* in the WD0513 region of the genome (30, 31) (forward primer (SEQ ID NO:5): 5'-CAAATTGCTCTTGTCCTGTGG-3', reverse primer (SEQ ID NO:6): 5'-GGGTGTTAAGCAGAGTTACGG-3'), Probe Cy5 (SEQ ID NO:7): TGAAATGGAAAAATTGGCGAGGTGTAGG- BHQ3. The second primer set binds to the polymorphic insertion sites of *wMelPop-CLA* and *wMelCS* at loci *IS5-WD1310* (forward primer (SEQ ID NO:8): 5'-CTCATCTTTACCCGTAATAAAATTTC-3', reverse primer (SEQ ID NO:9): 5'-TCTTCCTCATTAAGAACCTCTATCTTG-3'), Probe HEX (SEQ ID NO:10): TAGCCTTTTACTTGTTTCCGGACAACCT-BHQ1. For each sample, quantitative PCR amplification of DNA was performed using a LightCycler 480 II Instrument (Roche) using LightCycler 480 SYBR Green I Master (Roche) for *wPip* and *wRi* amplification and Taqman Probes Master for amplifying *wMelCS* according to the manufacturer's protocol.

Wolbachia density and distribution

The density of *Wolbachia* in the three newly introduced strains, *w*MelCS, *w*Ri and *w*Pip was compared to the density in the long established *w*MelF strain (14, 15). Total relative *Wolbachia* densities for the four lines were determined in whole, female mosquitoes using qPCR using primers to amplify a fragment of the gene coding 16S rRNA (16S) (forward primer (SEQ ID NO:11): 5'-GAGTGAAGAAGGCCTTTGGG-3', reverse primer (SEQ ID NO:12): 5'-CACGGAGTTAGCCAGGACTTC-3', probe (SEQ ID NO:13): 5' LC640-CTGTGAGTACCGTCATTATCTTCCTCACT-IowaBlackRQ-3') and the reference *Ae. aegypti rps17* gene (forward primer (SEQ ID NO:14): 5'-TCCGTGGT ATCTCCATCAAGCT-3', reverse primer (SEQ ID NO:15): 5'-CACTTCCGGCACGTAGTTGTC-3', probe (SEQ ID NO:16): 5'FAM-CAGGAGGAGGAACGTGAGCGCAG-BHQ1-3').

Vector competence

DENV-3 Cairns 08/09 strain stocks (Genbank accession number: JN406515.1) were prepared by inoculation of C6/36 cells with a multiplicity of infection (MOI) of 1 and collection of culture supernatant 6-7 days later. Virus concentrations were determined by TCID₅₀ as previously described (32) using monoclonal antibody 4G2 (33).

For feeding experiments with DENV-3 (Cairns 08/09) infected blood, 100 seven-day old age-controlled female mosquitoes were placed in 500 mL plastic containers (five containers per *Wolbachia* line, two containers per Tet line), starved for up to 24 h and allowed to feed on a 50:50 mixture of defibrinated sheep blood and tissue culture supernatant containing freshly harvested 2.0×10^6 TCID₅₀/mL of DENV-3. Feeding was done through a piece of desalted porcine intestine stretched over a water-jacketed membrane feeding apparatus preheated to 37°C. Mosquitoes were left to feed in the dark for approximately 80 min. Fully engorged mosquitoes were placed in 500 mL containers at a density of < 20/container, and incubated for 14 d at 26°C with 65% RH and a 12 h light/dark cycle.

For adult microinjections, 60 six or seven-day old age-controlled female mosquitoes were anesthetized by CO₂. The mosquitoes were injected intrathoracically with 69 nL of DENV-3 Cairns 08/09 strain, 2.5×10^6 TCID₅₀/ml (or 10, 100 or 1000-fold dilutions thereof) in RPMI media (Life Technologies) using a pulled-glass capillary and a handheld microinjector (Nanoject II, Drummond Scientific). Injected mosquitoes were incubated for 7 days (10 mosquitoes/cup) at 26°C with 65% RH and a 12 h light/dark cycle. To quantify DENV-3 genomic copies, total RNA was isolated from DENV-3 mosquitoes (entire mosquitoes for injection experiments, or head and bodies separately for blood fed mosquitoes) using the RNeasy 96 QIAcube HT kit (Qiagen). DENV-3 copies were quantified using one step qRT-PCR (LightCycler® Multiplex RNA Virus Master, Roche), using primers to the conserved 3'UTR: Forward (SEQ ID NO:17): 5'-AAGGACTAGAGGTTAGAGGAGACCC; Reverse (SEQ ID NO:18): 5'- CGTTCTGTGCCTGGAATGATG; Probe (SEQ ID NO:19): 5'- HEX- AACAGCATATTGACGCTGGGAGAGACCAGA-BHQ1-3'. Mosquito extracts with >1000 copies of DENV per well were scored positive, based on the LOD₉₅ (limit of detection 95%) for DENV-3 with this primer set.

RESULTS

20 *Maternal transmission and cytoplasmic incompatibility*

Maternal transmission of a *Wolbachia* strain and its ability to induce CI are key features that must be conserved when considering new *Wolbachia*-infected *Ae. aegypti* lines for field releases. CI occurs when an uninfected female mated with a *Wolbachia*-infected male cannot produce viable offspring. This phenomenon ensures all offspring will be *Wolbachia*-positive, enabling effective spread of *Wolbachia* throughout a wild population.

Maternal transmission was assayed by crossing *Wolbachia*-infected females with males from their respective tetracycline (Tet)-treated line (minimum generation 5). The crosses revealed near-complete maternal transmission for wMelCS (99.4%) and wPip (98.9%) while wRi sustained some breakdown of maternal transmission at 88% (Table 1).

Cytoplasmic incompatibility (CI) was determined for each line by measuring the hatch rate from crosses between Tet-treated females and infected males from each respective *Wolbachia*-infected line. Control crosses where Tet-treated males and females were mated or *Wolbachia*-infected males and females were mated showed high hatch rates. In CI crosses, near-complete sterility was observed in all three lines, comparable to previous reports for *wMel* (Fig. 1A) (14, 24).

Mosquito fitness costs induced by each Wolbachia strain

Fecundity, hatch rate and egg diapause viability

Fecundity was measured as the number of eggs collected/female following mating between *Wolbachia*-infected males and females, and *Wolbachia*-infected females and Tet-treated males, compared to the control cross of Tet-treated males and females. For *wRi* and *wMelCS*, the number of eggs/female did not significantly decrease irrespective of these matings indicative of high fecundity (Fig. 1B). For *wPip*, relative to control crosses, the mean number of eggs/female decreased for crosses between infected females and males, and infected females crossed with uninfected males, suggesting *wPip* reduces fecundity for this line.

We next examined the egg hatch rates of each cross. No significant reduction in hatch rate was observed for *wRi* crosses between *Wolbachia*-infected females and males, when compared to Tet-treated male and female crosses. *wMelCS* showed a slight although significant reduction in the hatch rate between *Wolbachia*-infected females and uninfected males, compared to the Tet-treated control cross (79% and 90%; $p < 0.05$) (Fig. 1A), similar to that reported for *wMel* (14, 24). Hatch rate was more dramatically impaired for *wPip*, with rates dropping from 82% for the Tet-treated control cross, to 58% for *Wolbachia*-infected male and female crosses ($p < 0.0001$).

To determine the effect of *Wolbachia* on egg viability and storage, eggs were collected and stored for 1-12 weeks before determining hatch rates. The benchmark transinfected line, *wMel* had a slight, but significant reduction in hatch rate following 4, 8, and 10 weeks storage ($p < 0.05$) compared to its Tet control

line, with a slightly larger reduction after 12 weeks ($p < 0.0001$) (Fig. 1C). *wRi* and *wMelCS* lines displayed similar hatch rates ($> 60\%$) across all ages until week 10 (no significant difference in hatch rates compared to the respective Tet control lines until week 8). By contrast, *wPip* eggs appeared to deteriorate rapidly, with $< 20\%$ hatch rate observed after 3 weeks of aging (significant reductions at all weeks tested, $p < 0.0001$, compared to *wPip.Tet*). Together, these data indicate a substantial fitness cost in the *wPip* line.

Longevity

Effects on the survival of mosquitoes from each line were next examined over time (Fig. 2). *wMel* females had the longest mean survival time (80 days), followed by *wMelCS* (56 days), then *wPip* (53 days) and finally *wRi* (51 days). Similar relative survival rates were observed for males, although all males had a shorter lifespan than their female counterparts as previously reported (24). Interestingly, *wMel*, *wMelCS* and *wRi* *Wolbachia*-infected lines tracked closely with their Tet-treated controls suggesting the infection had minimal influence on longevity, with the genetics of the generated line being a more important contributor. Minor, but significant differences were observed for *wRi* females ($p < 0.0001$) and *wMel* ($p < 0.05$) and *wMelCS* ($p < 0.05$) males, relative to their respective Tet control lines. *wPip* was the only line that showed substantially shorter lifespan for both males and females compared to the Tet-control ($p < 0.0001$). No line showed impaired longevity that would be of relevance in a field setting where $< 1\%$ of female *Ae. aegypti* are expected to live beyond 3 weeks (34).

Wolbachia density and distribution

Total *Wolbachia* density was measured in 5-day old adult females of each line (minimum generation 9) and compared to benchmark line *wMel*, using qPCR for primers specific to the highly conserved *Wolbachia* ribosomal RNA gene, *16S*, and *Ae. aegypti rps17* gene to normalize for DNA input. Total *Wolbachia* density in *wMelCS* and *wPip* lines was comparable to *wMel* (no significant

difference), while the mean *w*Ri density was approximately half that of *w*Mel ($p < 0.0001$; Fig. 3A).

The localization of *Wolbachia* within ovary and salivary gland tissues was next examined within adult female mosquitoes using fluorescence *in situ* hybridisation (FISH). Mosquitoes were formaldehyde-fixed, and paraffin-embedded tissue sections stained using a probe specific for *Wolbachia* 16S gene (red, Fig. 3B). Total DNA was stained with DAPI (blue). Consistent with the strong maternal transmission shown in Table 1 for all lines, each strain was abundant in ovarian tissues, with distribution comparable with *w*Mel (Fig. 3B). Similar distribution of *Wolbachia* was also observed for all lines in the salivary glands, key for restricting viral transmission.

Restriction of DENV-3 replication by wRi and wMelCS in Ae. aegypti

To compare the viral-blocking capacity of these lines, mosquitoes were fed an infectious blood meal containing 2.0×10^6 TCID₅₀/ml (DENV-3), then incubated for 14 days at 26°C. Due to the reduced fitness of *w*Pip mosquitoes we elected to exclude this line, and vector competence analysis was performed on *w*Ri and *w*MelCS lines, and compared to *w*Mel. Mosquitoes were collected, the head separated from each mosquito body, then total RNA extracted to measure rates of infection (as determined by bodies positive for DENV RNA) and dissemination (heads positive for DENV RNA). Consistent with previous findings, *w*Mel reduced the mean DENV RNA levels by 3log₁₀ in mosquito bodies (3×10^4 copies/body compared to 3×10^7 copies/body in the relevant Tet control line), as well as reducing the infection rate, with just 9% of *w*Mel mosquito bodies scoring positive for DENV infection compared to 94% in the matched Tet control line (where positive was defined as >1000 copies/body, see Materials & Methods; Fig. 4 and Table 2).

By contrast, *w*Ri caused only a slight, although significant ($p < 0.01$, Mann-Whitney test) reduction in viral copies (7.6×10^7 copies/body compared to 1.3×10^8 copies/body for the matched Tet control line) with no reduction in infection rate (96% compared to 98%, respectively). *w*MelCS gave a phenotype intermediate of *w*Mel and *w*Ri, whereby a wide spread in the number of DENV

copies/body was observed, with a mean of 1.5×10^7 RNA copies compared to 4.7×10^7 RNA copies for its matched Tet control line. Interestingly, DENV-3 dissemination, key for viral transmission, was similarly restricted between *wMel* and *wMelCS* lines, with $\sim 4\log_{10}$ reduction in viral RNA copies determined in the head of each line relative to the matched Tet control, and just 2 and 9% infection rates, respectively, compared to 83 and 96% for the Tet controls. While the mean DENV RNA copies/head of *wRi*-infected mosquitoes was significantly reduced (2×10^6 copies compared to 9.5×10^6 copies for the Tet control line), the dissemination rates were not reduced (94% compared to 95%, respectively).

Together, these results indicate that *wRi* demonstrates reduced DENV-blocking capacity while the ability of *wMelCS* to restrict viral dissemination appears to be similar to that of *wMel*.

In order to more closely examine the blocking capacity of *wMelCS* we next used an injection challenge model to directly compare viral restriction in *wMel* and *wMelCS* lines. Female mosquitoes were injected with 2.5×10^6 TCID₅₀/ml of DENV-3, or 10-fold dilutions thereof. Mosquitoes were incubated for 7 days before total RNA extraction from entire mosquitoes. When mosquitoes were injected with undiluted virus, both *Wolbachia* lines struggled to restrict viral replication with the number of DENV copies reduced by $< 1\log_{10}$ relative to each matched Tet control line (Fig 5 A and B). When mosquitoes were injected with 2.5×10^5 TCID₅₀/ml, *wMelCS* reduced DENV RNA copies by $\sim 2\log_{10}$ compared to $< 1\log_{10}$ for *wMel*, indicating that *wMelCS* may be more effective at restricting replication DENV-3 when mosquitoes are challenged with a highly infectious virus, compared to mosquitoes modified by another *Wolbachia* strain such as *wMel*. This trend continued as the virus was diluted further, with substantially lower viral RNA levels measured in *wMelCS* mosquitoes when injected with 2.5×10^4 TCID₅₀/ml and 2.5×10^3 TCID₅₀/ml. Importantly, lower rates of infection were also observed in *wMelCS* compared to *wMel* for the three lowest injected virus concentrations (“number of DENV-positive mosquitoes/total blood-fed mosquitoes” are indicated above each bar).

Overall, these data suggest that *wMelCS* could restrict a direct challenge

by DENV-3 more effectively than *wMel*.

DISCUSSION

Field releases of *wMel*-transinfected *Ae. aegypti* in order to establish
5 *Wolbachia* in wild populations have been expanding over the past 6 years in 4
countries endemic to DENV. This technology is now recommended by the World
Health Organisation for operational pilot deployments considering the ongoing
global Zika virus emergency (35). While cluster randomized trials measuring the
impact of this intervention on disease transmission are still underway,
10 observational studies suggest this is a highly promising approach and justifies
further investment to optimize *Wolbachia* strain selection.

Here we report for the first time the transinfection of *Ae. aegypti* with
Wolbachia strains *wRi*, *wMelCS* and *wPip*. *wRi* and *wMelCS*-infected mosquitoes
displayed similar fitness to *wMel*-infected mosquitoes. However, the introduction
15 of *wPip* lead to poor egg viability after short storage times, and weaker fecundity
and hatch rates compared to *wMel*, *wMelCS* and *wRi*; traits that would be likely
to compromise effective release strategies when performed on a large scale.
Interestingly, in *Ae. albopictus* transinfected with *wPip*, similar reductions in
female fecundity and egg hatch rate were observed (36), suggesting that *wPip*
20 may broadly induce this fitness cost when introduced into a non-native host.
Although this may be context dependent, as work from Zhang *et al.* (2015)
determined no fitness cost to *Ae. albopictus* when *wPip* was introduced into
mosquitoes that also contained the native *wAlbA* and *wAlbB* strains (37).

When tested for their ability to block DENV, *wMelCS* and *wRi* restricted
25 replication and dissemination following an infectious blood meal, although *wRi*
blocking was substantially reduced compared to *wMelCS*, with *wMelCS*
restricting DENV dissemination to the mosquito head to a similar extent as *wMel*.
A virus injection challenge model where mosquitoes are essentially overloaded
with virus, was used to separate the ability of *wMel* and *wMelCS* to inhibit DENV
30 in a more infectious setting, with *wMelCS* demonstrating superior blocking under
all concentrations of virus tested. While viral infection of mosquitoes by blood

feeding may be considered a more physiologically relevant model in which to examine virus blocking, the injection challenge model does provide information regarding the robustness of *Wolbachia*-mediated blocking and perhaps suggests that the *wMelCS* strain is better able to cope with high viral loads. This may be a critical feature in a clinical setting where viral loads in patients, and subsequent infection rates in mosquitoes occur at far greater levels than in artificial blood feeding systems (38).

It has been inferred that the degree of blocking correlates with density of the *Wolbachia* strain in key tissues (39-41). We show here that overall *Wolbachia* densities are nearly identical in *wMelCS*-transinfected mosquitoes compared to *wMel*, despite *wMelCS* appearing to have a stronger virus-blocking phenotype. Although analysis of ovarian and salivary gland tissues by FISH indicated similar amounts of *Wolbachia* for each line, tissue-specific quantitative analysis of *wMelCS* may identify differential densities and localization of *Wolbachia* in other tissues that contribute to virus blocking, e.g. nervous tissue (42).

In *wRi*-containing mosquitoes, *Wolbachia* was found to be present at approximately half the overall density of *wMel*, and was significantly worse at blocking DENV-3 replication and dissemination. Interestingly, when Osborne *et al.* (2009) examined blocking of DCV by *wRi* and transinfected-*wMel* in *D. simulans* they observed almost identical densities of *wRi* and *wMel*, similar protection against virus-induced mortality to flies infected with DCV, yet *wRi* did not reduce the viral load (19). While this supports a role for utilising *Wolbachia* densities to predict viral protection in mosquitoes, it highlights the complex nature of this tripartite interaction.

It should be noted that the apparent increase in DENV protection provided to *wMelCS*-infected mosquitoes, compared to *wMel*, is in line with work performed in *D. melanogaster* and *D. simulans* using DCV and FHV (21, 22), indicating that *Wolbachia*-infected *Drosophila* lines may be useful indicators for pathogen blocking in *Wolbachia*-infected mosquitoes.

With no substantial fitness cost identified in *wMelCS* infections, this strain may prove to have higher DENV blocking characteristics in the field than *wMel*

without the fitness costs that prevent field establishment of wMelPop-CLA (18). To test this prediction these mosquitoes now need to be examined in more realistic challenge settings, such as blood feeding on viremic patients.

Throughout this specification, the aim has been to describe the preferred
5 embodiments of the invention without limiting the invention to any one embodiment or specific collection of features. Various changes and modifications may be made to the embodiments described and illustrated herein without departing from the broad spirit and scope of the invention.

All computer programs, algorithms, patent and scientific literature referred
10 to herein is incorporated herein by reference in their entirety.

Table 1. *Wolbachia* presence in the progeny from crosses between infected females and uninfected males.

	No. Parent Females	No. Female progeny screened	No. <i>Wolbachia</i> positive
wMelCS	37	651	647 (99.4%)
wRi	54	1253	1096 (87.5%)
wPip	49	434	429 (98.9%)

Table 2. *Restriction of DENV-3 infection and dissemination by Wolbachia strains wRi and wMelCS.*

Line	Body		Head	
	Number DENV +	% DENV +	Number DENV +	% DENV +[#]
wMel	22 (250)	9	5 (250)	2
wMel.Tet	78 (83)	94	69 (83)	83
wMelCS	135 (240)	56	21 (240)	9
wMelCS.Tet	74 (76)	97	73 (76)	96
wRi	166 (169)	98	159 (169)	94
wRi.Tet	103 (107)	96	102 (107)	95

[#] Calculated as a percentage of total engorged mosquitoes

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CLAIMS

1. A mosquito infected with a mosquito-adapted wMelCS bacterium and optionally infected with a highly infectious virus.

2. A population of mosquitoes that includes one or more mosquitoes
5 infected with a highly infectious virus and one or more mosquitoes that comprise mosquito-adapted wMelCS bacteria.

3. A method of modifying an environment that comprises mosquitoes comprising a highly infectious virus capable of being transmitted by one or more of the mosquitoes in the environment, including the step of introducing
10 one or more mosquitoes that comprise mosquito-adapted wMelCS bacteria into the environment to thereby modify the environment.

4. A method of modifying a mosquito population including the step of introducing one or more mosquitoes that comprise mosquito-adapted wMelCS bacteria into the mosquito population to thereby modify the mosquito
15 population, wherein the mosquito population comprises mosquitoes that harbour a highly infectious virus capable of being transmitted by one or more mosquitoes to a vertebrate host.

5. A method of at least partly suppressing or inhibiting transmission of a highly infectious virus within a vertebrate host population having one or
20 more individuals harbouring the highly infectious virus, including the step of introducing one or more mosquitoes that comprise mosquito-adapted wMelCS bacteria into an environment comprising the vertebrate host population to thereby at least partly suppress or inhibit transmission of the highly infectious virus within the vertebrate host population.

25 6. A method of at least partly preventing, reducing or eliminating a disease caused by, or associated with, a highly infectious mosquito-borne virus, said method including the step of introducing one or more mosquitoes that comprise mosquito-adapted wMelCS bacteria into an environment where the highly infectious mosquito-borne virus is, or may become, present to
30 thereby at least partly prevent, reduce or eliminate the incidence of the disease in said environment.

7. The method of Claim 6, wherein the environment comprises a vertebrate host or host population.

8. The mosquito of Claim 1, the population of Claim 2 or the method of any one of Claim 3-8, wherein the mosquito-adapted *wMelCS* bacteria do not produce or elicit a substantial fitness cost to *wMelCS*-infected mosquitoes.

9. The mosquito, population or method of Claim 8, wherein the fitness cost includes impaired egg viability and larval development, although without limitation thereto.

10. The mosquito of Claim 1 or Claim 8, the population of Claim 2 or Claim 8 or method of any one of Claims 3-9, wherein the mosquito-adapted *wMelCS*-infected mosquitoes display substantially repressed or inhibited virus replication, substantially normal maternal transmission and substantially normal cytoplasmic incompatibility (CI).

11. The mosquito of any one of Claims 1 or 8-10, the population of any one of Claims 2 or 8-10 or the method of any one of Claims 3-10, wherein the mosquito is *Aedes aegyptii*.

12. The mosquito of any one of Claims 1 or 8-11, the population of any one of Claims 2 or 8-11 or the method of any one of Claims 3-11, wherein the virus is dengue virus, Zika virus or chikungunya virus.

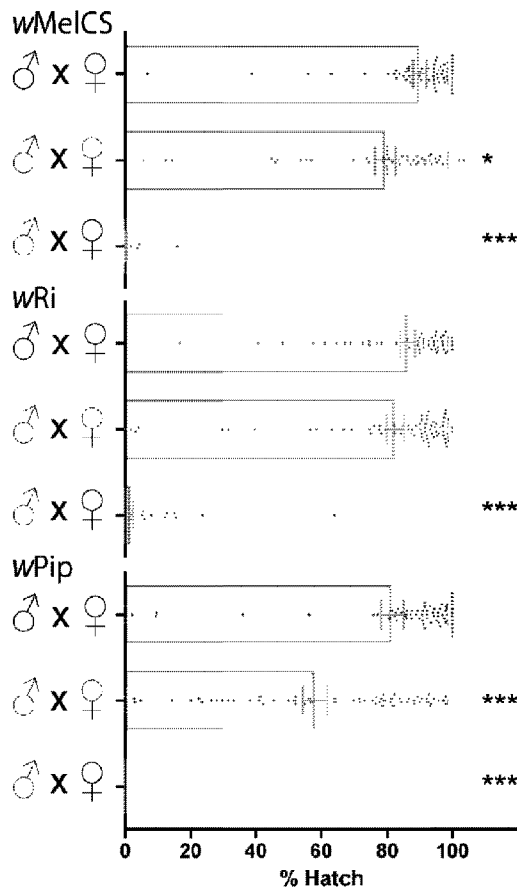
13. The mosquito of any one of Claims 1 or 8-12, the population of any one of Claims 2 or 8-12 or the method of any one of Claims 3-12, wherein the highly infectious virus corresponds to, or is equivalent to, a viral load or titre in a vertebrate host that can result in a virus transmission rate to a mosquito that feeds on the vertebrate host of at least 30%.

14. The mosquito of any one of Claims 1 or 8-13, the population of any one of Claims 2 or 8-13 or the method of any one of Claims 3-13, wherein the mosquito-adapted *wMelCS* bacterium is *Wolbachia pipientis* var *wMelCS* deposited at the National Measurement Institute, Port Melbourne, Victoria, Australia under accession number V17/025608 on 14 November 2017.

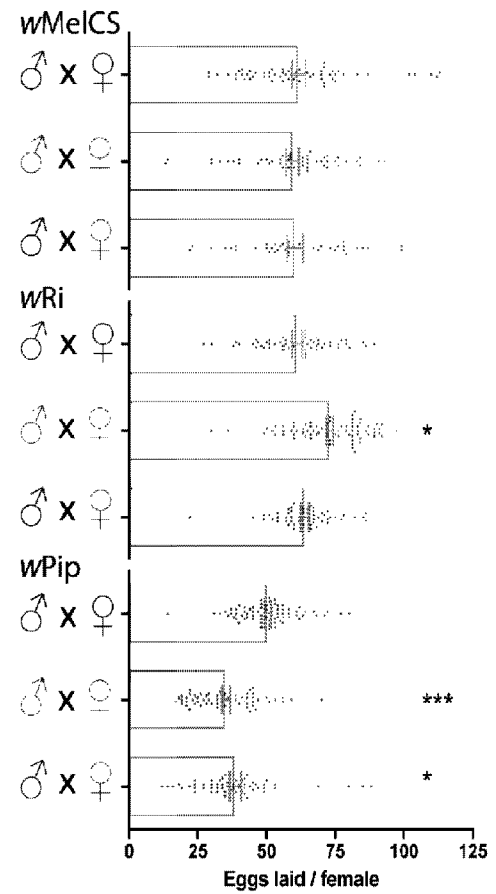
15. A mosquito-adapted bacterium which is *Wolbachia pipientis* var *wMelCS* deposited at the National Measurement Institute, Port Melbourne,

Victoria, Australia under accession number V17/025608 on 14 November 2017.

A



B



C

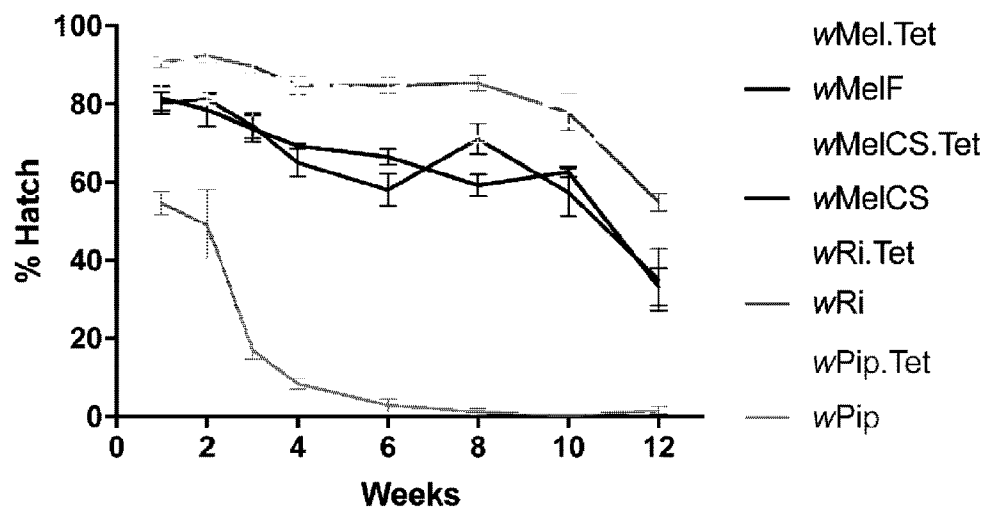
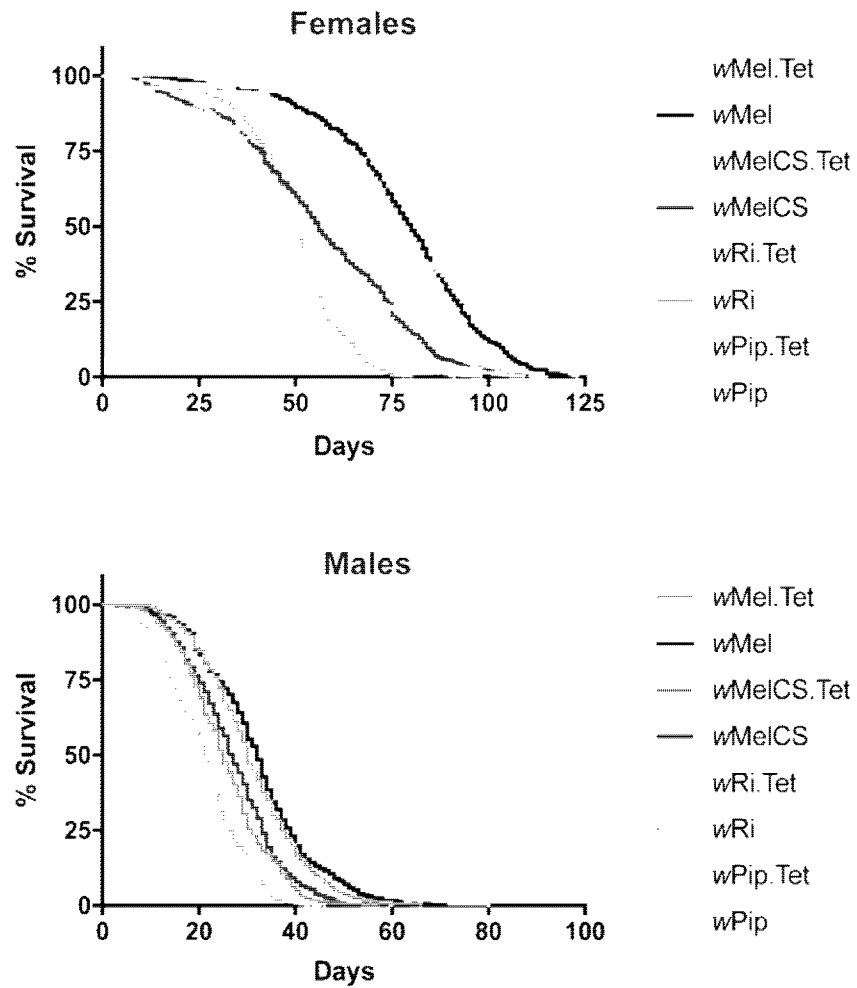
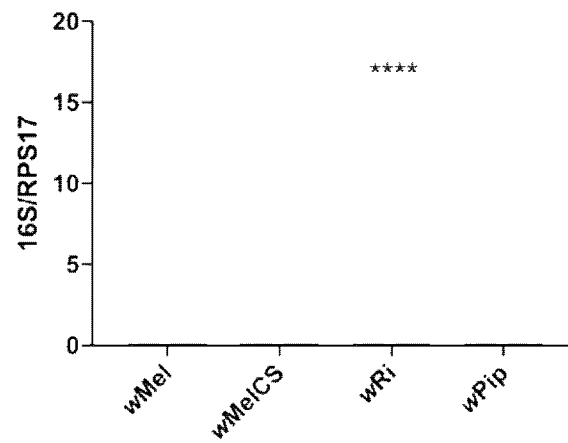


Figure 1

**Figure 2**

A



B

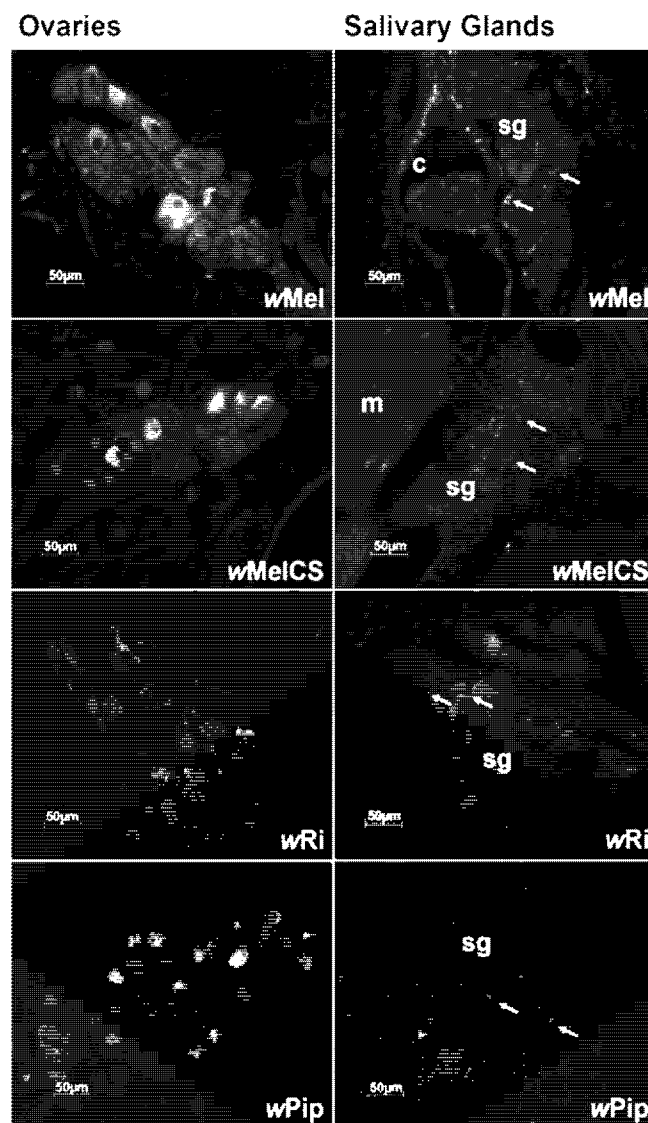


Figure 3

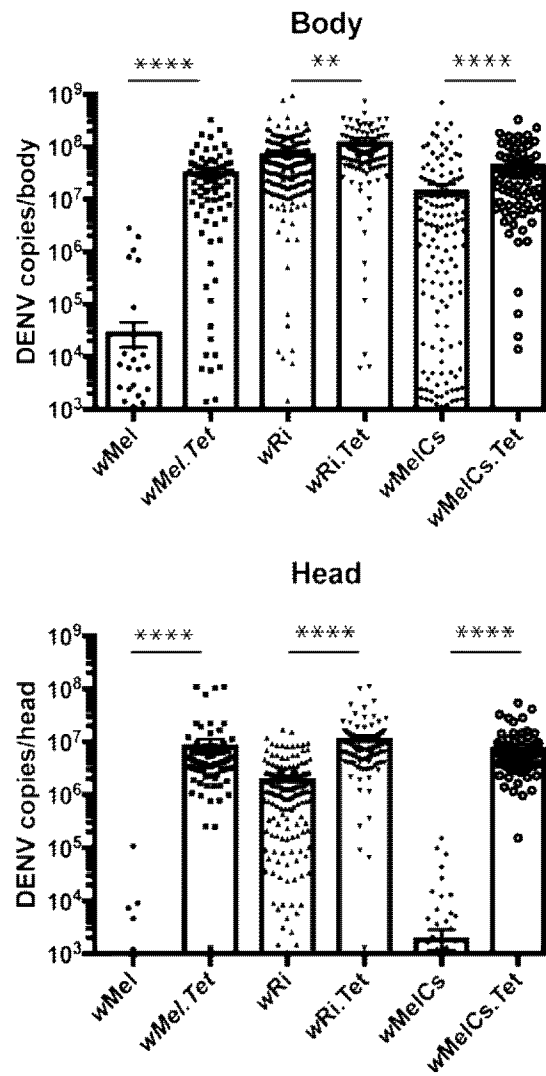
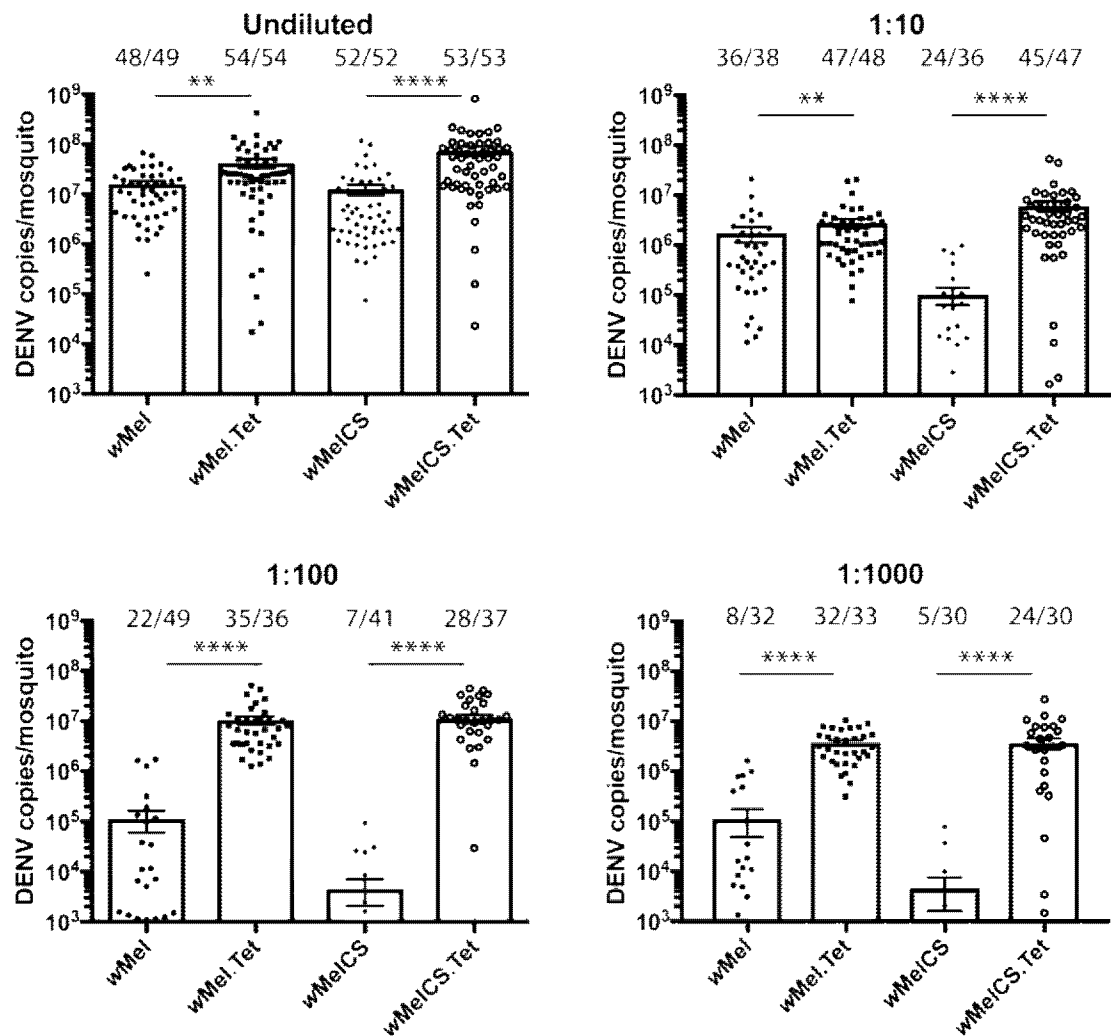


Figure 4

A



B

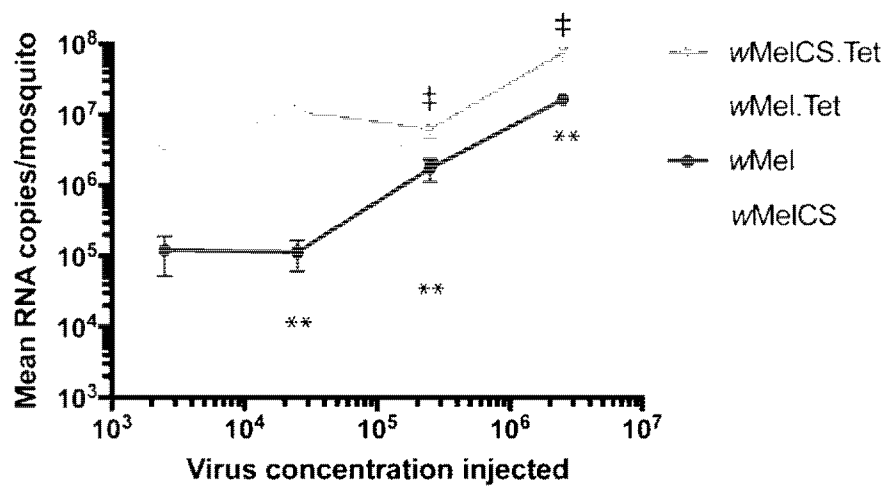


Figure 5

INTERNATIONAL SEARCH REPORT

International application No.
PCT/AU2018/050643

A. CLASSIFICATION OF SUBJECT MATTER

A01K 67/033 (2006.01) A01N 63/00 (2006.01) C12N 1/20 (2006.01)

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

PATENW, CAPLUS, BIOSIS, EMBASE, MEDLINE & keywords: Wolbachium pipientis, wMeICS, mosquito, virus and like terms.

AUSPAT, ESPACENET, PUBMED, Internal databases: inventor and applicant names

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
	Documents are listed in the continuation of Box C	

☒ Further documents are listed in the continuation of Box C

☒ See patent family annex

* "A"	Special categories of cited documents: document defining the general state of the art which is not considered to be of particular relevance	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"E"	earlier application or patent but published on or after the international filing date	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"O"	document referring to an oral disclosure, use, exhibition or other means	"&"	document member of the same patent family
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search
20 August 2018

Date of mailing of the international search report
20 August 2018

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INTERNATIONAL SEARCH REPORT		International application No.
C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		PCT/AU2018/050643
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 2011/0145939 A1 (O'NEILL) 16 June 2011 Abstract; [0109], [0110], [0120]-[0121], [0153], Examples	15
A	WO 2013/026994 A1 (ISIS INNOVATION LIMITED) 28 February 2013 Abstract; page 2 line 1 – page 5 line 4	
X	CHROSTEK, E. et al., “ <i>Wolbachia</i> Variants Induce Differential Protection to Viruses in <i>Drosophila melanogaster</i> : A Phenotypic and Phylogenomic Analysis”, PLOS Genetics, 2013, vol. 9, no. 12, e1003896 Results	15
P,X	Fraser J. E. et al., “Novel <i>Wolbachia</i> -transinfected <i>Aedes aegypti</i> mosquitoes possess diverse fitness and vector competence phenotypes, 7 December 2017, PLoS Pathogens, vol. 13(12):e1006751. whole document	1-15

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INTERNATIONAL SEARCH REPORT Information on patent family members		International application No. PCT/AU2018/050643	
This Annex lists known patent family members relating to the patent documents cited in the above-mentioned international search report. The Australian Patent Office is in no way liable for these particulars which are merely given for the purpose of information.			
Patent Document/s Cited in Search Report		Patent Family Member/s	
Publication Number	Publication Date	Publication Number	Publication Date
US 2011/0145939 A1	16 June 2011	US 2011145939 A1	16 Jun 2011
		AU 2009222557 A1	13 Jan 2011
		AU 2009222557 B2	11 Jun 2015
		CA 2707880 A1	17 Dec 2010
		US 2014298501 A1	02 Oct 2014
		US 9090911 B2	28 Jul 2015
WO 2013/026994 A1	28 February 2013	WO 2013026994 A1	28 Feb 2013
End of Annex			
Due to data integration issues this family listing may not include 10 digit Australian applications filed since May 2001. Form PCT/ISA/210 (Family Annex)(January 2015)			