Genome evolution of dengue virus serotype 1 under selection by *Wolbachia pipientis* in *Aedes aegypti* mosquitoes

Short title: Dengue virus evolution in *Wolbachia*-infected mosquitoes

Duong Thi Hue Kien[1], Kathryn M Edenborough[2,6#], Daniela da Silva Goncalves[2], Tran Thuy Vi[1], Etiene Casagrande[2,5], Huynh Thi Le Duyen[1], Vo Thi Long[1], Le Thi Dui[1], Vu Thi Tuyet Nhu[1], Nguyen Thi Giang[1], Huynh Thi Xuan Trang[1], Elvina Lee[2], I'ah Donovan-Banfield[2], Huynh Thi Thuy Van[1], Nguyen Minh Nguyet[1], Nguyen Thanh Phong[4], Nguyen Van Vinh Chau[4], Bridget Wills[1,3], Sophie Yacoub[1], Heather Flores[2,5], Cameron P Simmons[2,6#]

[1]Oxford University Clinical Research Unit, Hospital for Tropical Disease, Ho Chi Minh City, Vietnam

[2]World Mosquito Program, Institute of Vector-Borne Disease, Monash University, Clayton, VIC, Australia

[3]Nuffield Department of Medicine, University of Oxford, Oxford, United Kingdom.

[4]Hospital for Tropical Diseases, 190 Ben Ham Tu, District 5, Ho Chi Minh City, Vietnam

[5]School of Biological Sciences, Monash University, Clayton, VIC, Australia

[6]Department of Microbiology, Biomedicine Discovery Institute, Monash University, Clayton, VIC, Australia

[#]Corresponding authors;

Cameron Simmons. E-mail: cameron.simmons@worldmosquito.org

Kathryn Edenborough. E-mail: kat.edenborough@monash.edu

Abstract

The introgression of antiviral strains of *Wolbachia* into *Ae. aegypti* mosquito populations is a public-health intervention for the control of dengue. Plausibly, dengue virus (DENV) could evolve to bypass the antiviral effects of *Wolbachia* and undermine this approach. Here, we established a serial-passage system to investigate evolution of dengue virus in *Ae. aegypti* mosquitoes infected with the *w*Mel strain of *Wolbachia*. Using this system, we report on virus genetic outcomes after twenty passages of serotype 1 of dengue virus (DENV-1). An amino acid substitution, E203K, in the DENV-1 envelope protein was more frequently detected in the consensus sequence of virus populations passaged in *w*Mel-infected *Ae. aegypti* than wild-type counterparts. Positive selection at residue 203 was reproducible; it occurred in passaged virus populations from independent DENV-1-infected patients, and also in a second, independent experimental system. In wild-type mosquitoes and human cells, the 203K variant was rapidly replaced by the progenitor sequence. These findings provide proof of concept that *w*Mel-associated selection of virus populations can occur in experimental conditions. Field-based studies are needed to explore whether *w*Mel imparts selective pressure on DENV evolution in locations where *w*Mel is established.

Introduction

Dengue is a public health burden on many tropical countries and is responsible for significant morbidity and healthcare expenditure (Martelli et al. 2015; Stanaway et al. 2016; Wilastonegoro et al. 2020). Deployment of *Wolbachia*-infected mosquitoes is one evidence-based, public-health approach that locally controls dengue (Indriani et al. 2020; Nazni et al. 2019; O'Neill et al. 2018; Pinto et al. 2021; Ryan et al. 2019; Utarini et al. 2021). Developed and implemented by the World Mosquito Program, the replacement approach has established *Wolbachia* in *Ae. aegypti* populations within cities and towns in regions with high dengue incidence: Asia, Latin America and Oceania.

The approach harnesses the collective host effects of *Wolbachia*, an endosymbiont that can rapidly introgress into mosquito populations by virtue of maternal transmission and cytoplasmic incompatibility (CI) (Ross et al. 2017; Sinkins 2004; Walker et al. 2011; Xi et al. 2005). The main *Wolbachia* strain deployed in the field (*w*Mel) confers protection to *Ae. aegypti* – and indirectly protects humans – from medically-important arboviruses, including all four serotypes of dengue virus (DENV1-4) (Carrington et al. 2018; Fraser et al. 2017; Frentiu et al. 2014; Moreira et al. 2009; Walker et al. 2011). DENV-1 is marginally less inhibited by *w*Mel than the other three serotypes (Carrington et al. 2018; Ferguson et al. 2015; Flores et al. 2020). Plausibly, incomplete viral inhibition might spawn variants, accelerating virus evolution.

The lifespan of the approach might hinge on the stability of the tripartite relationship between *Ae. aegypti*, *Wolbachia* and dengue virus. Interestingly, the *w*Mel genome is highly stable when introgressed into *Ae. aegypti* populations, with limited evolution after several years in the field (Dainty et al. 2021; Huang et al. 2020; Ross et al. 2022). In

contrast, DENV1-4 are positive-sense, single-stranded RNA viruses with naturally mutable genomes attested by the divergence of multiple viral lineages within each serotype (Holmes and Twiddy 2003; Twiddy et al. 2003).

One inherent risk then, to the success of the *Wolbachia* introgression approach, is the emergence of DENV variants that escape the virus-specific inhibition in *w*Mel-infected mosquitoes. Escape variants might be selected if they bypass the multifactorial intracellular modifications induced by *Wolbachia* infection, such as changes to lipid content, and alteration of the cytoskeleton and endoplasmic reticulum membranes (Edenborough et al. 2021; Geoghegan et al. 2017; A. Lindsey et al. 2018). These organelles are critical for DENV replication (Heaton and Randall 2010; Junjhon et al. 2014; Perera et al. 2012; Samsa et al. 2009), such that the mutational requirements for escape might lead to viral fitness trade-offs. Unfit DENV variants are likely to be removed from the virus population via purifying selection (Holmes 2003) and might be thus challenging to identify directly in the field.

Understanding if *w*Mel can exert selective pressure on the DENV genome under experimental conditions might help to identify escape variants and facilitate surveillance in the field. Thus, this study compared the viral genetic variants that evolved during serial passage of DENV-1 in *w*Mel-infected versus wild-type *Ae. aegypti.* With this system, *w*Mel-induced positive selection of amino acid residues in the DENV-1 envelope (E) was observed.

Results

Adaptive evolution of DENV-1 in *w*Mel-mosquitoes features positive selection of codons in the DENV-1 envelope

We developed an *in vivo* DENV serial-passage system and used it to study virus evolution in the presence of *w*Mel. The system involved oral-feeding cohorts of wild-type (WT) and *w*Mel-infected *Ae. aegypti* with viremic blood from DENV-1 (genotype I) infected adult dengue patients, in the Hospital for Tropical Diseases in Ho Chi Minh City (HCM), Vietnam.

A total of 6 acute viremic blood samples from 6 independent dengue patients established 18 cohorts of WT mosquitoes (3 replicate cohorts per patient) and 18 cohorts of *w*Mel-infected *Ae. aegypti* (3 replicate cohorts per patient) with a HCM genetic background. Ten days after blood feeding, the virus populations in each cohort of mosquitoes were serially passaged twenty times, via intrathoracic injection with a 7-day incubation period, through their cognate mosquito line. The schematic in **Fig 1** represents the serial passage system. Using a large volume of virus for passaging resulted in comparable viral genome copies between mosquito lines (*w*Mel and WT) for all patients and passages (P0-P20) and genome copies were sufficient for whole genome sequencing (**Fig S1)**. *w*Mel density in the mosquito cohorts was also tested and remained stable between passage numbers (**Fig S2**).

We hypothesised that virus single nucleotide variants (SNVs) providing a selective advantage in the presence of *w*Mel would increase in frequency over passage in *w*Mel and not WT-mosquito cohorts, and ultimately appear in the consensus sequence (> 50% frequency) (Lequime et al. 2017). By P20, two SNVs had met our predefined temporal and 50% prevalence thresholds i.e. present in more than half of the total

mapped reads in a cohort and increased temporally between P0 and P20. The two SNVs were located at 1447 and 1990 in the envelope (E) coding region. The 1447G>A SNV was detected at > 50% frequency in 7 of 18 *w*Mel-mosquito cohorts originating from four of the total six independent patient plasma samples (**Fig 2** and **Table S1**). In contrast, 1447G>A was not detected above 50% in any WT-mosquito cohort at any passage number from any patient plasma sample. When observed in WT-mosquitoes, 1447G>A was detected only transiently and at low frequency. The negative and positive controls included in our passage system and sequencing pipeline suggested low-level detection of 1447A in WT-mosquitoes was unlikely to result from cross-sample contamination (**Table S5**), however we cannot exclude this possibility altogether. The 1990G>A SNV was present at > 50% frequency in 9 of 18 *w*Mel- and 13 of 18 WT-mosquito cohorts in parallel across all six independent patient samples (**Fig 3 and Table S1**).

SNVs 1447G>A and 1990G>A resulted in E→K substitutions in the DENV envelope protein at amino acid residues 203 and 384 of the E protein, respectively. Over-representation of E203K in the *w*Mel-mosquito line might suggest it is involved in adaption to *w*Mel. The emergence of E384K in both mosquito lines suggests this mutation might hold a general advantage for DENV-1 infection in mosquitoes. Interestingly, the 1447G>A and 1990G>A were not present with ≥ 50% frequency in the same cohort of mosquitoes, raising the possibility of a fitness cost when both variants are present in a virus population.

Stability of 1447A (203K) in mosquitoes in the presence and absence of *w*Mel selective pressure

We hypothesised that the frequency of 1447A would decline in the absence of *w*Mel selection. To test this we serially passaged P20 virus populations from *w*Mel mosquito cohorts from patients 172, 180 and 185 ten times in WT and *w*Mel mosquito cohorts. Each of these virus populations contained a > 50% frequency of the 1447A variant. We report on 1447A and G frequencies in each mosquito cohort at P21, P25 and P30 in **Fig 4** and **Table S2**. After one passage in WT-mosquitoes (P21), 1447A frequencies remained above 50% consensus levels, yet these declined by P25, plummeting to < 10% in all three virus lineages by P30, with the 1447A variant being replaced by the wild-type progenitor sequence (1447G) (**Fig 4A**).

Passage of the same three P20 supernatants revealed 1447A persisted over 10 passages in *w*Mel-infected mosquitoes; 1447A became fixed (> 99%) by P30 in all three lineages (**Fig 4B**). These findings reinforced the concept of *w*Mel being the driving force underpinning 1447A (E203K) selection and suggest 1447A was negatively selected in the absence of *w*Mel.

Transience of 1447A (203K) and 1990A (384K) in human cell lines

DENV variants that are unfit or unstable in human cells, might pose a reduced risk of establishing in the mosquito-human transmission cycle. We thus measured 203K and 384K variant frequency during DENV passage in human cells. In THP-1 and K-562 human cell lines, 1447A frequencies declined rapidly (< 10%) within 5 passages for all three of the virus lineages that were passaged. Reductions in 1447A were due to reversion to 1447G as the consensus within the virus population (**Fig 4C-D, Table S2**). We also quantified DENV genome copies for whole virus populations (containing 1447A and 1447G) to ascertain the magnitude of DENV infection in human cells. Mean DENV

genome copies of three *w*Mel-adapted virus lineages were equivalent to mean levels measured during passage of three progenitor virus lineages, and overall, DENV copy numbers increased with passage number (**Table S3)**. This finding indicated variant fluctuation did not result from poor susceptibility of the cell lines to DENV-1 infection.

We next asked if the rapid frequency decline in human cells was a specific observation for 1447A, or common to other variants uncovered in our passaging study. In human cells, we passaged three supernatants from P20 wildtype-mosquito lineages from patients 172, 180 and 185 that contained high frequencies of 1990A; the SNV selected in both *w*Mel- and WT-mosquito cohorts. Similar to 1447A, 1990A also declined in frequency to < 10% by P5 (**Fig 4E-F**). These findings suggest 1447A (203K) and 1990A (384K) are not sufficiently competitive to persist in the consensus sequence for multiple rounds of infection in human cells.

*w*Mel selection is reproducible with a different DENV-1 strain

To assess the robustness of the findings related to 1447G>A and 1990G>A SNVs, we replicated the serial passage study using a tissue-culture grown DENV-1 genotype I isolate (DV1/Vietnam/2008) instead of viremic blood and a different mosquito line (Australian *w*Mel-infected *Ae. aegypti* and an uninfected control line, cured from *w*Mel infection with tetracycline).

We observed higher *w*Mel densities in Australian mosquitoes than in the HCM mosquitoes used previously (**Fig S2**). Differences in density could result from differential processing of Australian mosquito supernatants, which were not filtered to retain as much virus as possible for passage. Potentially these unfiltered supernatants from Australian mosquitoes contained more cellular debris, which may account for the

increased *Wolbachia* density detected. Nevertheless, we observed DENV-1 genome copies in Australian and HCM mosquito cohorts that were similar (**Fig S1** and **Fig S3**).

In support of our previous findings, the 1447G>A (E203K) and 1990G>A (E384K) SNVs developed *denovo* during the repeat serial passage experiment. As we expected to observe these substitutions *a priori*, we report their frequency even when below the previously predefined 50% threshold. The 1447G>A SNV was first identified at P15 in *w*Mel-mosquitoes at a mean frequency of 31.8% ± 7.2 in two of three mosquito cohorts and was identified again in one cohort at P20 (16.6%) (**Fig 5** and **Table S4**). In the control mosquito line, 1447G>A arose in one P10 cohort at 34% but was not present at P15 nor P20. Sporadic emergence of 1447G>A in control mosquito lines is consistent with prior data (**Fig 2**). At the same codon, a 1448A>G SNV (E203G) was detected in one *w*Mel-mosquito cohort at P15 (86.2%) and P20 (35.0%).

We identified an additional six non-synonymous substitutions that increased in frequency temporally. However, excluding one conservative substitution (A96V) in NS4B, all other substitutions were detected in both mosquito lines (**Fig 5**). This included the 1990G>A (E384K) substitution in DENV-1, which was detected at P20 in one *w*Mel mosquito cohort and all three control mosquito cohorts at a mean frequency of 40.3%. The appearance of substitutions (E203K and E384K) in both experiments, although observed at lower frequencies in the 2nd replicate experiment, highlights the overall reproducibility of these mutations in the context of a different mosquito genetic background and virus strain.

Discussion

The likelihood of DENV evolving resistance to the antiviral potency of *w*Mel is an ill-defined risk to the *Wolbachia* introgression method. Here, we report DENV-1 genetic outcomes from a mosquito serial-passage system that identifies signals of *w*Mel-associated selection. The passage system defined amino acids in E under positive selection in *w*Mel-infected (203 and 384) and WT-mosquitoes (384).

The substitutions (E203K and E384K) arose independently in replicate cohorts, during passage of DENV that was acquired from individual DENV-1 positive patients. In a replicate experiment, where the DENV-1 strain and mosquito genetic background differed, both substitutions also arose within several technical replicate mosquito cohorts.

The recurring nature of 203 and 384 substitutions in this study might result from their centralized position within homopolymeric (HP) A tracts in the viral genome. HP tracts are sites at which RNA-dependent RNA polymerases readily induce mutations (Olspert et al. 2016; Ratinier et al. 2008).

Despite its recurrence, E203K was only weakly selected by *w*Mel in the replicate experiment. *w*Mel does not localize to all mosquito tissues and its distribution can be focal (Amuzu et al. 2018; Fraser et al. 2020), hence some somatic cells of the mosquito are likely to be *Wolbachia*-free. Inter-experimental variation might be due to the stochastic nature of variant and wildtype virus competition for replication in *Wolbachia*-free cells of the mosquito. Alternatively, E203K might impart fitness costs on different DENV-1 genetic backbones, therein limiting its selective advantage.

Putative effects of E203K and E384K for DENV infection and *w*Mel blocking

DENV genome sequences encoding 203K, are rarely observed (< 5%) in public viral genome repositories (92% of 4100 sequences are 203E). As a consequence of low prevalence, the function of 203K in viral infection has not been studied for the DENV-1 serotype.

The E→K substitution at residue 203, located in domain II of E, would increase local positive charge and position cationic sidechains proximal to a conserved histidine (261H) based on the DENV-1 E 3D structure. Histidine-cation interactions are known as pH-sensing residues (Kudlacek et al. 2021) that trigger dimer-to-trimer rearrangement of E via electrostatic repulsion (Chaudhury et al. 2015; Kampmann et al. 2006; Kuhn et al. 2015; Modis et al. 2003). We thus speculate that the 203 E→K change, in the vicinity of 261H might impact the kinetics of E trimerization and thus pH thresholds of fusion.

Hypothetically, the E203K substitution could modify fusion pH thresholds, such that the 203K variant might display atypical traversal of the endocytic pathway. We speculate that modified fusion thresholds could result in uncoating within early rather than late endosomes. Fusion with early endosomes might be beneficial for viral entry into *w*Mel-infected cells, as *w*Mel perturbs lipid homeostasis, causing defects in lipid processing within late endosomes (Geoghegan et al. 2017). Sufficient lipid concentration in late endosomes is crucial for DENV uncoating, and potentially lipid dysregulation might inhibit this process (Zaitseva et al. 2010). It is important to recognise that the ability of the E203K variant to evade *w*Mel-induced virus blocking has not been studied here. This substitution might constitute the first step in the selection process, where *w*Mel-induced blocking is multifactorial and likely requires the accumulation of several mutations in the viral genome.

We speculate that the E384K variant, detected in WT- and *w*Mel-infected mosquitoes may provide a general advantage for viral attachment to mosquito cells at 26-28 degrees. Residue 384 is located within DIII of E, is highly surface exposed and is part of a 10 amino acid loop that mediates binding to mosquito but not mammalian cells for DENV-2 (Hung et al. 2004). Four of these ten residues (382-385) are described in mosquito-borne but not tick-borne flaviviruses suggesting they may determine host tropism (Modis et al. 2003). Binding to mosquito cells in a constant temperature environment (26-28 degrees) might select for E proteins with specific thermodynamic requirements, and limited exposure to human receptors might select for variants that bind mosquito receptors with high affinity.

Virus passage studies and *Wolbachia*; limitations and relevance to the field

Key elements that set our passage system apart from prior studies (Koh et al. 2019; Martinez et al. 2019) include the use of oral feeding or a large intrathoracic (IT) injection volume to establish infection in mosquitoes. Delivery of small volumes is standard practice for IT injection but might bottleneck genetic diversity in the virus population, and likely contributed to *w*Mel-driven viral extinction in past passage studies in *D. melanogaster* (Martinez et al. 2019). IT injection also bypasses midgut infection and escape barriers (Forrester et al. 2012) and its use might have altered the selective pressures in our passage system, and the breadth of adaptive variants we thus observed. One difference though is we used large injection volumes to deliver high virus doses, which has been shown to limit *w*Mel-mediated blockade of virus replication (Fraser et al. 2017). We link the use of a large bolus of viral inoculum, derived from

whole mosquito bodies, to the interesting finding that typical *w*Mel-induced blocking of viral load was not observed.

Ultimately, we aim to determine the risk that 203K and 384K variants present to the *Wolbachia* method. Although both variants arise reproducibly in these passage experiments, they also readily revert to their progenitor forms (203E and 384E) in human cells, and at least in the case of 203, in WT-mosquitoes. Transcience of 203K and 384K in human cells might limit the risk of positive selection as DENV cycles between two hosts. Applying these results to the field, it is likely 203K and 384K would be removed by virtue of purifying selection during mosquito-to-human transmission. Continued study of 203K and 384K variant fitness (i.e. kinetics of dissemination and transmission in mosquitoes), which we have not determined here, will provide more insight into the risk of these variants persisting in natural transmission cycles.

In summary, this study identified an amino acid change in the DENV-1 E protein that is reproducibly selected during passage in *w*Mel-infected *Ae. aegypti*. The results demonstrate proof of concept that *w*Mel can impart selective pressure on the virus population in laboratory conditions. Whether such variants could evolve, or are already present at low frequency in locations where *Wolbachia* has been introgressed into the *Ae. aegypti* population is unknown. Nonetheless, these laboratory-based detections of virus evolution could be used to inform prospective DENV population genetic studies in *Wolbachia* release sites.

Materials and Methods

Ethics Statement and Dengue Patient Cohorts

Mosquito colonies were blood-fed on adult, human volunteers in accordance with Monash University Human Research Ethics permit number CF11/0766-2011000387. Written informed consent was given by all volunteers before commencing. For blood feeding of mosquito colonies in Vietnam, adult volunteers provided written informed consent before their participation.

The study involving patient blood samples was carried out at the Oxford University Clinical Research Unit, Ho Chi Minh City (HCM), Vietnam. It included feeding mosquitoes with viremic blood collected from six febrile dengue patients. The relevant ethical protocols were reviewed and approved by the Scientific and Ethical Committee of the Hospital of Tropical Diseases (HTD) (EC CS/NĐ/16/27) and the Oxford Tropical Research Ethical Committee (OxTREC Reference: 45-16). Patients included in the study provided written informed consent, were ≥ 15 year of age, inpatients at HTD with < 96 h fever and confirmed positive for DENV infection with an NS1 rapid test.

Biosecurity and biosafety measures

At Monash University, mosquito infections were conducted within a quarantine insectary (AA-V2165) that operates at biosecurity containment level 3. The mutations identified in this serial passage experiment are not known to increase pathogenicity and have been approved for recombinant virus production by the Office of the Gene Technology Regulator under the licence number DNIR-639.

Virus serial-passage method

Derivation of mosquitoes and rearing

Aedes aegypti mosquitoes used in experiments were of either HCM or Australian (Cairns) genetic backgrounds. Australian *w*Mel and tetracycline-treated mosquito

control lines were generated as previously described (Fraser et al. 2017; Walker et al. 2011). The HCM *w*Mel mosquito line was produced by backcrossing Australian *w*Mel onto a HCM background and outcrossing the colonies as previously described (Carrington et al. 2018). All mosquitoes were maintained at 26-28 °C, 65-85% RH, and a 12:12 h light:dark cycle, with access to 10% sucrose solution *ad libitum*. The HCM mosquitoes used in the experiments were from F_{25}-F_{46} and G_{28}-G_{49} for wildtype and *w*Mel mosquitoes respectively. Australian mosquitoes used in the experiments were from G_{37}-G_{41} and G_{44}-G_{48} for *w*Mel and control lines respectively.

Initial exposure of mosquitoes to viremic patient blood

Patient blood samples were collected in EDTA tubes and confirmed to contain DENV-1 with a serotype-specific qPCR assay as previously described (Hue et al. 2011). Blood samples were fed to mosquitoes directly after collection to establish infection for passage 0. WT and *w*Mel females (2 to 4 d old) were orally fed for 30 mins using artificial membrane feeders containing 300 µL of the patient's blood. Mosquitoes were cold anaesthetized at 4 °C for 45 s and engorged females were transferred to 500 mL plastic containers. They were maintained in an environmental chamber at 27 °C with 12:12 h light:dark cycle and 65-85% RH.

Initial exposure of mosquitoes to tissue-culture derived DENV

TVP/22776/Vietnam/2008 DENV-1 (GenBank accession FJ461335) was sourced from the World Reference Centre for Emerging Viruses and Arboviruses (WRCEVA) and amplified for two passages in C6/36 cells. Virus stocks were sequenced to confirm their identity and to generate a full-length genome for reference mapping. DENV-1 stock (1.2

× 10^6 $TCID_{50}$/ml) was diluted 1:5 in RPMI media and injected intrathoracically (69 nL) into mosquitoes to establish passage 0 mosquito cohorts.

Mosquito collection into cohorts

Seven - fourteen days post-exposure, mosquitoes were collected into RPMI media containing 2% fetal calf serum (Gibco), 2 mM L-glutamine (Gibco), 2.5 µg/mL Fungizone, 50 U/mL Penicillin - 50 µg/mL Streptomycin (Gibco). Mosquitoes were homogenized in media with a silica bead beater for 3 mins at 30 Hz and the supernatant was clarified by centrifugation for 1 min, 1,400 RCF and passed through a 0.2 µm filter (Millipore). Supernatants (50 µl/mosquito) from 20 mosquitoes were combined to produce one cohort.

At each passage, triplicate cohorts were produced for each patient and mosquito line. Each cohort was maintained as an independent lineage over passage. For the passage experiment using tissue-culture grown virus, mosquitoes were homogenized with a stainless-steel bead at 30 Hz for 1 min with a TissueLyser II instrument (Qiagen). The supernatant was clarified via two centrifugation steps (5000 RCF for 10 mins at 4 °C) rather than filtration.

Mosquito infection for serial passage

For passages 1-20, twenty naïve mosquitoes were inoculated per cohort by intrathoracic injection with 0.5-1 µL of pooled supernatant. We performed 20 cycles of passaging of DENV in *w*Mel-infected or uninfected control *Ae. aegypti*. We also established negative (mosquitoes not injected with DENV-1) and positive (mosquitoes injected with a distinct DENV-1 strain Accession Number: FJ432735) controls to detect cross-sample contamination. The controls were run alongside test samples and

assayed at passages 1, 5, 10, 15 and 20 according to the PCR and sequencing pipelines outlined below.

Virus and *Wolbachia* genome quantification

Viral RNA in patient blood and mosquito cohorts was extracted using either MagNA Pure 96 DNA and Viral NA Small Volume Kit (Roche) on the MagNA Pure 96 System or using QIAamp viral RNA Kit (Qiagen) and quantified using previously described RT-qPCR assays (Fraser et al. 2017; Hue et al. 2011). The *Wolbachia* infection status in wildtype and *w*Mel infected females was confirmed by relative quantification of the *Wolbachia wsp* gene to the *Ae. aegypti rps17* reference gene as previously described (Fraser et al. 2017). All qPCR was performed using a LightCycler 480 II Instrument (Roche).

Viral whole-genome sequencing

Extracted RNA was subjected to cDNA synthesis using SuperScript IV First-Strand Synthesis System (Thermo Fisher Scientific) according to manufacturer instructions. Tiled amplification of the whole DENV genome was conducted in two separate multiplex PCR reactions with Q5 high-fidelity DNA polymerase (New England Biolabs) using previously described conditions (Quick et al. 2017).

The amplicons were purified with AMPure XP beads (Beckman Coulter) and pooled at equal concentrations, which were determined with the PicoGreen dsDNA quantitation assay (Beckman Coulter). Pooled DNA of 0.4 ng/μL were used as input for further library construction using Nextera XT DNA Library Preparation Kit (Illumina). Index samples were quantified with KAPA Library Quantification kit (Roche) and then pooled together at 1.5-2 nM. The pooled library was sequenced with MiSeq v2 300 or v3 600

cycle reagent (Illumina). Passage samples and controls were pooled into six independent sequencing libraries, which were sequenced in separate runs as indicated by sequencing batch ID in **Table S5**.

NGS Analysis

Amplification and Illumina sequencing of DENV genomes was performed on triplicate cohorts of mosquitoes at P0, P10, P15 and P20. The total number of reads mapped to the reference sequence, coverage depth and Q scores of viral genomes in passage material and controls are summarised in **Table S5** and **S6**.

Generating reference sequences for mapping

Sequence reads from the initial oral feed (passage 0) were assembled *de novo* with CLC Genomics Workbench 9.0 or Geneious Prime 2021.1.1. The longest contiguous sequence was searched in the nr/nt nucleotide collection of Genbank, in order to select the reference genome, which presented the highest homology. Sequence reads were then re-mapped to the homologous DENV-1 sequence to generate a full-length genome sequence suitable for reference-based mapping. For the passage experiment using tissue-culture grown virus, reference sequences were generated by sequencing DENV-1 virus stocks.

Generating consensus sequences and SNV calling

Adapter trimming and fastq demultiplexing were performed with default Illumina pipelines. Further analyses were performed with CLC (for the patient-derived virus passage experiment) and a custom snakemake pipeline (for the tissue-culture derived virus passage experiment). In CLC, read ends were trimmed based on a quality score

limit of 0.05 and ambiguous (N) characters were removed with a limit of 2 allowed per read. Quality scores were calculated on a Phred scale and converted to base-calling error probability. Consensus sequences for the mosquito cohorts (passages 0, 10, 15 and 20) were achieved by mapping sequence reads to the passage 0 references for each cognate patient. The consensus sequence incorporates nucleotides with > 10× of sequencing depth and variant frequency level > 50%.

To identify regions in read mappings with unexpectedly low or high coverage, the coverage analysis was run. The algorithm fits a Poisson distribution to the observed coverages in the positions of the mapping. The two parameters, "Minimum length", and the "P-value threshold value" were set at 50 and 0.0001 respectively.

Low-frequency variants were detected using a Neighbourhood Quality Standard (NQS) model. For inclusion in the analysis, the minimum coverage cut-off was 1,000 and the per cent of minimum variant frequency cut-off was 0.5%. Viral genomes in some mosquito cohorts did not meet the criteria of high coverage and were excluded from the study and included patients #172: passage 15 (1 *w*Mel-mosquito cohort) & 20 (2 *w*Mel-mosquito cohorts), #176: passage 10 (1 *w*Mel-mosquito cohort), 15 (1 *w*Mel-mosquito cohort) and 20 (2 *w*Mel-mosquito cohorts) and #193: passage 10 (1 WT-mosquito cohort), 15 (1 WT-mosquito cohort) & 20 (1 WT-mosquito cohort).

For the tissue-culture derived virus passage experiment, viral cDNA, tiled PCRs and libraries were prepared in duplicate for each mosquito cohort. DENV primer sequences were removed from 5' and 3' read ends with Alien Trimmer (Criscuolo and Brisse 2013). Reads were also trimmed to remove low-quality bases (quality < 30) and small reads (length < 50 nucleotides). Trimmed reads were mapped to reference DENV genomes

with bwa-mem (Heng Li and Durbin 2009) and SNVs above a 10% threshold were called with iVar (Grubaugh et al. 2019). Only variants that were present in duplicated libraries were included for further analysis. Sequence data is deposited at National Center for Biotechnology Information under accession numbers MN912109.1-MN912248.1 and BioProject PRJNA898886.

Human cell infection with DENV-1 variants

Five serial passages of each DENV-1 variant (either 203K or 384K in envelope) were conducted in THP-1 and K-562 cells. Cells (10^5) were washed in RPMI with 2% FBS, exposed to 203K and 384K variants in a total volume of 0.2 ml, and incubated at 37°C for 2 hours with agitation every 20 minutes to prevent cell sedimentation. Cells were then washed six times by centrifugation (900 RCF for 3 min) and resuspended in fresh media, finally seeding cells in 24-well plates for 7 days of incubation at 37 °C with 5% CO_2. Supernatants were collected for four consecutive passages. Quantification of viral load by RT-qPCR and genome sequencing were performed at passages 1, 3 and 5.

Statistical analyses

Analyses were conducted and plotted in RStudio (version 1.2.5033), R (version 3.4.4) and GraphPad Prism (version 8).

Acknowledgements

We thank the patients and their families for their participation in this study and the staff in Ward D of the HTD.

Data availability

The raw data is available in the supplementary material

Funding

This work was supported by the World Mosquito Program (WMP) and the National Health and Medical Research Council, Australia, Investigator Grant 1173928.

References

Amuzu, H. E., Tsyganov, K., Koh, C., Herbert, R. I., Powell, D. R., & McGraw, E. A. (2018). '*Wolbachia* enhances insect-specific flavivirus infection in *Aedes aegypti* mosquitoes', *Ecology and Evolution*, 8/11: 5441–54. DOI: 10.1002/ece3.4066

Carrington, L. B., Tran, B. C. N., Le, N. T. H., Luong, T. T. H., Nguyen, T. T., Nguyen, P. T., Nguyen, C. V. V., et al. (2018). 'Field- and clinically derived estimates of *Wolbachia* -mediated blocking of dengue virus transmission potential in *Aedes aegypti* mosquitoes', *Proceedings of the National Academy of Sciences*, 115/2: 361–6. National Academy of Sciences. DOI: 10.1073/pnas.1715788115

Chaudhury, S., Ripoll, D. R., & Wallqvist, A. (2015). 'Structure-based pKa prediction provides a thermodynamic basis for the role of histidines in pH-induced conformational transitions in dengue virus', *Biochemistry and Biophysics Reports*, 4: 375–85. Elsevier B.V. DOI: 10.1016/j.bbrep.2015.10.014

Criscuolo, A., & Brisse, S. (2013). 'AlienTrimmer: A tool to quickly and accurately trim off multiple short contaminant sequences from high-throughput sequencing reads', *Genomics*, 102/5–6: 500–6. Academic Press. DOI: 10.1016/j.ygeno.2013.07.011

Dainty, K. R., Hawkey, J., Judd, L. M., Pacidônio, E. C., Duyvestyn, J. M., Gonçalves, D. S., Lin, S. Y., et al. (2021). '*w*Mel *Wolbachia* genome remains stable after 7 years in Australian *Aedes aegypti* field populations', *Microbial Genomics*, 7/9. England. DOI: 10.1099/mgen.0.000641

Edenborough, K. M., Flores, H. A., Simmons, C. P., & Fraser, J. E. (2021). 'Using *Wolbachia* to Eliminate Dengue: Will the Virus Fight Back?', *Journal of Virology*, 95/13: e0220320. American Society for Microbiology. DOI: 10.1128/jvi.02203-20

Ferguson, N. M., Hue Kien, D. T., Clapham, H., Aguas, R., Trung, V. T., Bich Chau, T. N., Popovici, J., et al. (2015). 'Modeling the impact on virus transmission of *Wolbachia* -mediated blocking of dengue virus infection of *Aedes aegypti*', *Science Translational Medicine*, 7/279: 279ra37. American Association for the Advancement of Science. DOI: 10.1126/scitranslmed.3010370

Flores, H. A., Taneja de Bruyne, J., O'Donnell, T. B., Tuyet Nhu, V., Thi Giang, N., Thi Xuan Trang, H., Thi Thuy Van, H., et al. (2020). 'Multiple *Wolbachia* strains provide comparative levels of protection against dengue virus infection in *Aedes aegypti*', *PLoS Pathogens*, 16/4: e1008433. Public Library of Science. DOI: 10.1371/journal.ppat.1008433

Forrester, N. L., Guerbois, M., Seymour, R. L., Spratt, H., & Weaver, S. C. (2012). 'Vector-Borne Transmission Imposes a Severe Bottleneck on an RNA Virus Population', *PLoS Pathogens*, 8/9: e1002897. DOI: 10.1371/journal.ppat.1002897

Fraser, J. E., de Bruyne, J. T., Iturbe-Ormaetxe, I., Stepnell, J., Burns, R. L., Flores, H. A., & O'Neill, S. L. (2017). 'Novel Wolbachia-transinfected Aedes aegypti mosquitoes possess diverse fitness and vector competence phenotypes', *PLoS*

Pathogens, 13/12: e1006751. Public Library of Science. DOI: 10.1371/journal.ppat.1006751

Fraser, J. E., O'Donnell, T. B., Duyvestyn, J. M., O'Neill, S. L., Simmons, C. P., & Flores, H. A. (2020). 'Novel phenotype of *Wolbachia* strain wPip in *Aedes aegypti* challenges assumptions on mechanisms of Wolbachia-mediated dengue virus inhibition', *PLoS Pathogens*, 16/7: e1008410. Public Library of Science (PLoS). DOI: 10.1371/journal.ppat.1008410

Frentiu, F. D., Zakir, T., Walker, T., Popovici, J., Pyke, A. T., van den Hurk, A., McGraw, E. A., et al. (2014). 'Limited Dengue Virus Replication in Field-Collected Aedes aegypti Mosquitoes Infected with Wolbachia', *PLoS Neglected Tropical Diseases*, 8/2: e2688. Public Library of Science. DOI: 10.1371/journal.pntd.0002688

Geoghegan, V., Stainton, K., Rainey, S. M., Ant, T. H., Dowle, A. A., Larson, T., Hester, S., et al. (2017). 'Perturbed cholesterol and vesicular trafficking associated with dengue blocking in Wolbachia-infected Aedes aegypti cells', *Nature Communications*, 8/1: 526. Nature Publishing Group. DOI: 10.1038/s41467-017-00610-8

Grubaugh, N. D., Gangavarapu, K., Quick, J., Matteson, N. L., de Jesus, J. G., Main, B. J., Tan, A. L., et al. (2019). 'An amplicon-based sequencing framework for accurately measuring intrahost virus diversity using PrimalSeq and iVar', *Genome Biology*, 20/1: 8. DOI: 10.1186/s13059-018-1618-7

Heaton, N. S., & Randall, G. (2010). 'Dengue Virus-Induced Autophagy Regulates Lipid Metabolism', *Cell Host & Microbe*, 8/5: 422–32. Cell Press. DOI: 10.1016/j.chom.2010.10.006

Holmes, E. C. (2003). 'Patterns of Intra- and Interhost Nonsynonymous Variation Reveal Strong Purifying Selection in Dengue Virus', *Journal of Virology*, 77/20: 11296–8. American Society for Microbiology. DOI: 10.1128/jvi.77.20.11296-11298.2003

Holmes, E., & Twiddy, S. (2003). 'The origin, emergence and evolutionary genetics of dengue virus', *Infection, Genetics and Evolution*, 3/1: 19–28. DOI: 10.1016/S1567-1348(03)00004-2

Huang, B., Yang, Q., Hoffmann, A. A., Ritchie, S. A., van den Hurk, A. F., & Warrilow, D. (2020). '*Wolbachia* Genome Stability and mtDNA Variants in Aedes aegypti Field Populations Eight Years after Release', *iScience*, 23/10: 101572. Elsevier Inc. DOI: 10.1016/j.isci.2020.101572

Hue, K. D. T., Tuan, T. V., Thi, H. T. N., Bich, C. T. N., Anh, H. H. le, Wills, B. A., & Simmons, C. P. (2011). 'Validation of an internally controlled one-step real-time multiplex RT-PCR assay for the detection and quantitation of dengue virus RNA in plasma', *Journal of Virological Methods*, 177/2: 168–73. Elsevier B.V. DOI: 10.1016/j.jviromet.2011.08.002

Hung, J.-J., Hsieh, M.-T., Young, M.-J., Kao, C.-L., King, C.-C., & Chang, W. (2004). 'An External Loop Region of Domain III of Dengue Virus Type 2 Envelope Protein Is Involved in Serotype-Specific Binding to Mosquito but Not Mammalian Cells', *Journal of Virology*, 78/1: 378–88. American Society for Microbiology. DOI: 10.1128/jvi.78.1.378-388.2004

Indriani, C., Tantowijoyo, W., Rancès, E., Andari, B., Prabowo, E., Yusdi, D., Ansari, M. R., et al. (2020). 'Reduced dengue incidence following deployments of *Wolbachia*-

infected *Aedes aegypti* in Yogyakarta, Indonesia: a quasi-experimental trial using controlled interrupted time series analysis', *Gates Open Research*, 4: 50. F1000 Research Ltd. DOI: 10.12688/gatesopenres.13122.1

Junjhon, J., Pennington, J. G., Edwards, T. J., Perera, R., Lanman, J., & Kuhn, R. J. (2014). 'Ultrastructural Characterization and Three-Dimensional Architecture of Replication Sites in Dengue Virus-Infected Mosquito Cells', *Journal of Virology*, 88/9: 4687–97. American Society for Microbiology. DOI: 10.1128/jvi.00118-14

Kampmann, T., Mueller, D. S., Mark, A. E., Young, P. R., & Kobe, B. (2006). 'The Role of Histidine Residues in Low-pH-Mediated Viral Membrane Fusion', *Structure*, 14/10: 1481–7. Cell Press. DOI: 10.1016/j.str.2006.07.011

Koh, C., Audsley, M. D., di Giallonardo, F., Kerton, E. J., Young, P. R., Holmes, E. C., & McGraw, E. A. (2019). 'Sustained *Wolbachia*-mediated blocking of dengue virus isolates following serial passage in *Aedes aegypti* cell culture', *Virus Evolution*, 5/1: vez012. DOI: 10.1093/ve/vez012

Kudlacek, S. T., Metz, S., Thiono, D., Payne, A. M., Phan, T. T. N., Tian, S., Forsberg, L. J., et al. (2021). 'Designed, highly expressing, thermostable dengue virus 2 envelope protein dimers elicit quaternary epitope antibodies.', *Science advances*, 7/42: eabg4084. American Association for the Advancement of Science. DOI: 10.1126/sciadv.abg4084

Kuhn, R. J., Dowd, K. A., Beth Post, C., & Pierson, T. C. (2015). 'Shake, rattle, and roll: Impact of the dynamics of flavivirus particles on their interactions with the host', *Virology*, 479–480: 508–17. Academic Press Inc. DOI: 10.1016/j.virol.2015.03.025

Lequime, S., Richard, V., Cao-Lormeau, V.-M., & Lambrechts, L. (2017). 'Full-genome dengue virus sequencing in mosquito saliva shows lack of convergent positive selection during transmission by Aedes aegypti', *Virus Evolution*, 3/2. Oxford University Press. DOI: 10.1093/ve/vex031

Li, Heng, & Durbin, R. (2009). 'Fast and accurate short read alignment with Burrows–Wheeler transform', *Bioinformatics*, 25/14: 1754–60. Oxford Academic. DOI: 10.1093/bioinformatics/btp324

Lindsey, A., Bhattacharya, T., Newton, I., & Hardy, R. (2018). 'Conflict in the Intracellular Lives of Endosymbionts and Viruses: A Mechanistic Look at Wolbachia-Mediated Pathogen-blocking', *Viruses*, 10/4: 141. Multidisciplinary Digital Publishing Institute (MDPI). DOI: 10.3390/v10040141

Martelli, C. M. T., Siqueira, J. B., Parente, M. P. P. D., Zara, A. L. de S. A., Oliveira, C. S., Braga, C., Pimenta, F. G., et al. (2015). 'Economic Impact of Dengue: Multicenter Study across Four Brazilian Regions', *PLoS Neglected Tropical Diseases*, 9/9: e0004042. Public Library of Science. DOI: 10.1371/journal.pntd.0004042

Martinez, J., Bruner-Montero, G., Arunkumar, R., Smith, S. C. L., Day, J. P., Longdon, B., & Jiggins, F. M. (2019). 'Virus evolution in *Wolbachia*- infected *Drosophila*', *Proceedings of the Royal Society B: Biological Sciences*, 286/1914: 20192117. Royal Society Publishing. DOI: 10.1098/rspb.2019.2117

Modis, Y., Ogata, S., Clements, D., & Harrison, S. C. (2003). 'A ligand-binding pocket in the dengue virus envelope glycoprotein', *Proceedings of the National Academy of Sciences*, 100/12: 6986–91. National Academy of Sciences. DOI: 10.1073/pnas.0832193100

Moreira, L. A., Iturbe-Ormaetxe, I., Jeffery, J. A., Lu, G., Pyke, A. T., Hedges, L. M., Rocha, B. C., et al. (2009). 'A Wolbachia Symbiont in Aedes aegypti Limits Infection with Dengue, Chikungunya, and Plasmodium', *Cell*, 139/7: 1268–78. DOI: 10.1016/j.cell.2009.11.042

Nazni, W. A., Hoffmann, A. A., NoorAfizah, A., Cheong, Y. L., Mancini, M. v., Golding, N., Kamarul, G. M. R., et al. (2019). 'Establishment of *Wolbachia* Strain *w*AlbB in Malaysian Populations of Aedes aegypti for Dengue Control', *Current Biology*, 29/24: 4241-4248.e5. Cell Press. DOI: 10.1016/j.cub.2019.11.007

Olspert, A., Carr, J. P., & Firth, A. E. (2016). 'Mutational analysis of the Potyviridae transcriptional slippage site utilized for expression of the P3N-PIPO and P1N-PISPO proteins', *Nucleic Acids Research*, 44/16: 7618–29. Oxford University Press. DOI: 10.1093/nar/gkw441

O'Neill, S. L., Ryan, P. A., Turley, A. P., Wilson, G., Retzki, K., Iturbe-Ormaetxe, I., Dong, Y., et al. (2018). 'Scaled deployment of Wolbachia to protect the community from dengue and other aedes transmitted arboviruses', *Gates Open Research*, 2: 36. F1000 Research Ltd. DOI: 10.12688/gatesopenres.12844.3

Perera, R., Riley, C., Isaac, G., Hopf-Jannasch, A. S., Moore, R. J., Weitz, K. W., Pasa-Tolic, L., et al. (2012). 'Dengue Virus Infection Perturbs Lipid Homeostasis in Infected Mosquito Cells', *PLoS Pathogens*, 8/3: e1002584. Public Library of Science. DOI: 10.1371/journal.ppat.1002584

Pinto, S. B., Riback, T. I. S., Sylvestre, G., Costa, G., Peixoto, J., Dias, F. B. S., Tanamas, S. K., et al. (2021). 'Effectiveness of Wolbachia-infected mosquito deployments in reducing the incidence of dengue and other Aedes-borne diseases in Niterói, Brazil: A quasi-experimental study', *PLoS Neglected Tropical Diseases*, 15/7: e0009556. Public Library of Science. DOI: 10.1371/journal.pntd.0009556

Quick, J., Grubaugh, N. D., Pullan, S. T., Claro, I. M., Smith, A. D., Gangavarapu, K., Oliveira, G., et al. (2017). 'Multiplex PCR method for MinION and Illumina sequencing of Zika and other virus genomes directly from clinical samples', *Nature Protocols*, 12/6: 1261–76. Nature Publishing Group. DOI: 10.1038/nprot.2017.066

Ratinier, M., Boulant, S., Combet, C., Targett-Adams, P., McLauchlan, J., & Lavergne, J.-P. (2008). 'Transcriptional slippage prompts recoding in alternate reading frames in the hepatitis C virus (HCV) core sequence from strain HCV-1', *Journal of General Virology*, 89/7: 1569–78. Microbiology Society. DOI: 10.1099/vir.0.83614-0

Ross, P. A., Robinson, K. L., Yang, Q., Callahan, A. G., Schmidt, T. L., Axford, J. K., Coquilleau, M. P., et al. (2022). 'A decade of stability for wMel Wolbachia in natural Aedes aegypti populations', *PLoS Pathogens*, 18/2: e1010256. Public Library of Science. DOI: 10.1371/journal.ppat.1010256

Ross, P. A., Wiwatanaratanabutr, I., Axford, J. K., White, V. L., Endersby-Harshman, N. M., & Hoffmann, A. A. (2017). '*Wolbachia* Infections in *Aedes aegypti* Differ Markedly in Their Response to Cyclical Heat Stress', *PLoS Pathogens*, 13/1: e1006006. Public Library of Science. DOI: 10.1371/journal.ppat.1006006

Ryan, P. A., Turley, A. P., Wilson, G., Hurst, T. P., Retzki, K., Brown-Kenyon, J., Hodgson, L., et al. (2019). 'Establishment of *w*Mel *Wolbachia* in *Aedes aegypti* mosquitoes and reduction of local dengue transmission in Cairns and surrounding locations in northern Queensland, Australia', *Gates Open Research*, 3: 1547. F1000 Research Ltd. DOI: 10.12688/gatesopenres.13061.1

Samsa, M. M., Mondotte, J. A., Iglesias, N. G., Assunção-Miranda, I., Barbosa-Lima, G., da Poian, A. T., Bozza, P. T., et al. (2009). 'Dengue Virus Capsid Protein Usurps Lipid Droplets for Viral Particle Formation', *PLoS Pathogens*, 5/10: e1000632. Public Library of Science. DOI: 10.1371/journal.ppat.1000632

Sinkins, S. P. (2004). '*Wolbachia* and cytoplasmic incompatibility in mosquitoes', *Insect Biochemistry and Molecular Biology*, 34/7: 723–9. Pergamon. DOI: 10.1016/j.ibmb.2004.03.025

Stanaway, J. D., Shepard, D. S., Undurraga, E. A., Halasa, Y. A., Coffeng, L. E., Brady, O. J., Hay, S. I., et al. (2016). 'The global burden of dengue: an analysis from the Global Burden of Disease Study 2013', *The Lancet Infectious Diseases*, 16/6: 712–23. DOI: 10.1016/S1473-3099(16)00026-8

Twiddy, S. S., Holmes, E. C., & Rambaut, A. (2003). 'Inferring the Rate and Time-Scale of Dengue Virus Evolution', *Molecular Biology and Evolution*, 20/1: 122–9. DOI: 10.1093/molbev/msg010

Utarini, A., Indriani, C., Ahmad, R. A., Tantowijoyo, W., Arguni, E., Ansari, M. R., Supriyati, E., et al. (2021). 'Efficacy of *Wolbachia*-Infected Mosquito Deployments for the Control of Dengue', *New England Journal of Medicine*, 384/23: 2177–86. Massachusetts Medical Society. DOI: 10.1056/NEJMoa2030243

Walker, T., Johnson, P. H., Moreira, L. A., Iturbe-Ormaetxe, I., Frentiu, F. D., McMeniman, C. J., Leong, Y. S., et al. (2011). 'The wMel Wolbachia strain blocks dengue and invades caged Aedes aegypti populations', *Nature*, 476/7361: 450–3. DOI: 10.1038/nature10355

Wilastonegoro, N. N., Kharisma, D. D., Laksono, I. S., Halasa-Rappel, Y. A., Brady, O. J., & Shepard, D. S. (2020). 'Cost of Dengue Illness in Indonesia across Hospital, Ambulatory, and not Medically Attended Settings', *The American Journal of Tropical Medicine and Hygiene*, 103/5: 2029–39. The American Society of Tropical Medicine and Hygiene. DOI: 10.4269/ajtmh.19-0855

Xi, Z., Khoo, C. C. H., & Dobson, S. L. (2005). 'Ecology: *Wolbachia* establishment and invasion in an *Aedes Aegypti* laboratory population', *Science (New York, N.Y.)*, 310/5746: 326–8. DOI: 10.1126/science.1117607

Zaitseva, E., Yang, S.-T., Melikov, K., Pourmal, S., & Chernomordik, L. v. (2010). 'Dengue Virus Ensures Its Fusion in Late Endosomes Using Compartment-Specific Lipids', *PLoS Pathogens*, 6/10: e1001131. Public Library of Science. DOI: 10.1371/journal.ppat.1001131

Fig 1: Schematic of DENV-1 passage system in WT- and *w*Mel- infected *Ae. aegypti*.
Six febrile dengue patients were enrolled into the study and their blood samples were fed to *Ae. aegypti* that were either wild-type (WT-mosquitoes) or infected with *Wolbachia* (*w*Mel-mosquitoes). Blood-feeding was used to establish DENV infection for the first passage (passage 0). Ten days after blood-feeding, *w*Mel and WT-mosquitoes were collected and processed to produce a virus-containing supernatant. For each patient sample and mosquito line, supernatants from 20 mosquitoes were combined to form three independent cohorts. The three cohorts were re-inoculation into the cognate mosquito line via intrathoracic (IT) injection and after seven days mosquitoes were collected and processed to produce virus-containing supernatant for the first passage (passage 1). The IT injection and collection process was repeated to passage 20. Supernatants from the three independent cohorts were processed separately and injected with fresh needles for each cohort to retain three independent replicates. Viral RNA extracted at passage 0, 10, 15 and 20 was subjected to qPCR and whole genome sequencing. This figure was created with BioRender.com

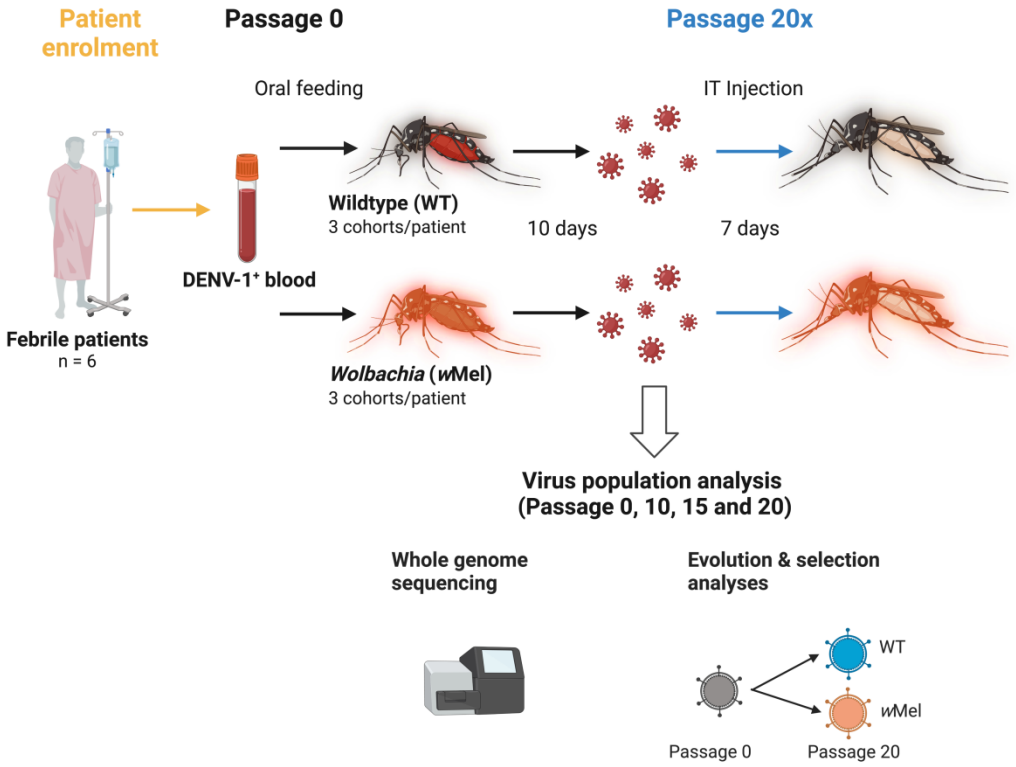

Fig 2: Evolution of the 1447G>A DENV-1 genomic variant in *w*Mel- and WT-mosquitoes. DENV-1 derived from patient blood was passaged 20 times in *w*Mel and WT-mosquitoes. One SNV, 1447G>A (E203K), increased in frequency between P0 and P20 and resulted in a consensus change of the DENV-1 genome (> 50% frequency). The SNV frequency (%) of the 1447G>A SNV in plasma and in *w*Mel (orange) or WT (blue) mosquito cohorts at P0, 10, 15 and 20 is shown per patient. Frequency is shown for individual mosquito cohorts by unique symbols. Cohorts negative for the 1447G>A SNV over the entire duration of passage are not shown.

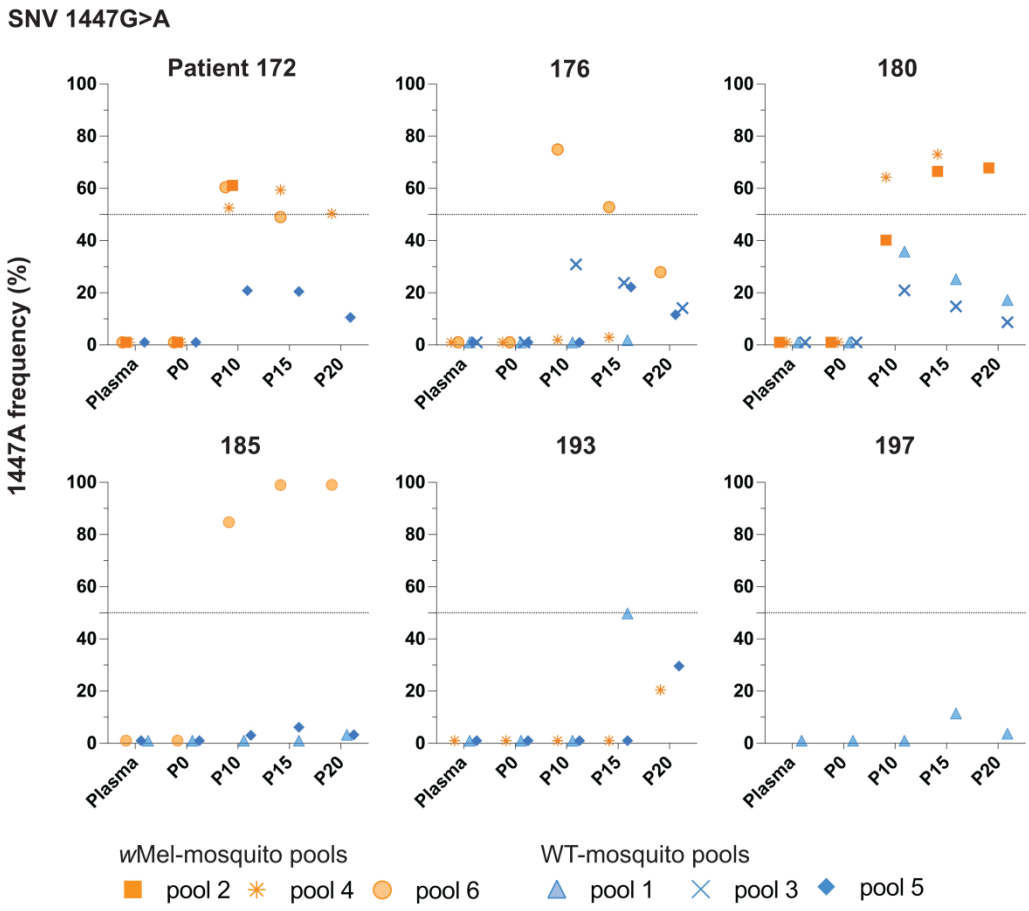

Fig 3: Evolution of the 1990G>A DENV-1 genomic variant in *w*Mel- and WT-mosquitoes. The 1990G>A (E384K) SNV resulted in a consensus change of the DENV-1 genome and increased in frequency between P0 and P20 in both mosquito lines. The SNV frequency (%) of 1990G>A SNV in plasma and *w*Mel (orange) or WT (blue) mosquito cohorts at P0, 10, 15 and 20 is shown per patient. Frequency is shown for individual mosquito cohorts by unique symbols. Cohorts negative for the 1990G>A SNV over the entire duration of passage are not shown.

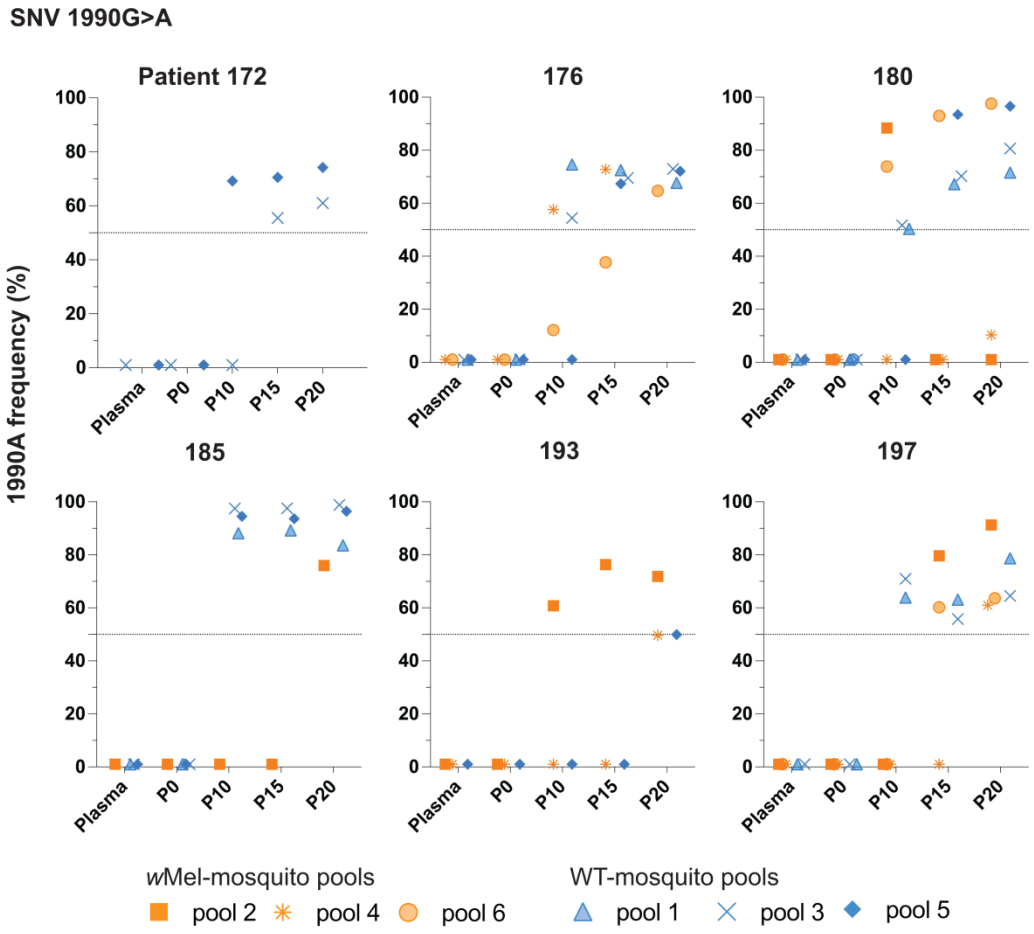

Fig 4: Decline in 1447A frequencies throughout passage in WT-mosquitoes and human cell lines.
*w*Mel-mosquito-adapted virus supernatants containing the 1447A variant at > 50% frequency were passaged 10x in WT (A) and *w*Mel-mosquitoes (B), and 5x in human cells lines: THP-1 (C) and K-562 (D). WT-mosquito-adapted virus supernatants containing 1990A at high frequencies were passaged 5x in THP-1 (E) and K-562 (F) cells. Bar charts show frequencies (% of reads containing each SNV of total reads) on the y-axis, which were determined by sequencing whole DENV genomes in passage 21, 25 and 30 mosquito cohorts, and passage 1, 3 and 5 virus supernatants from human cells. SNV frequencies of the G form is represented by dark colours and the A form by light colours.

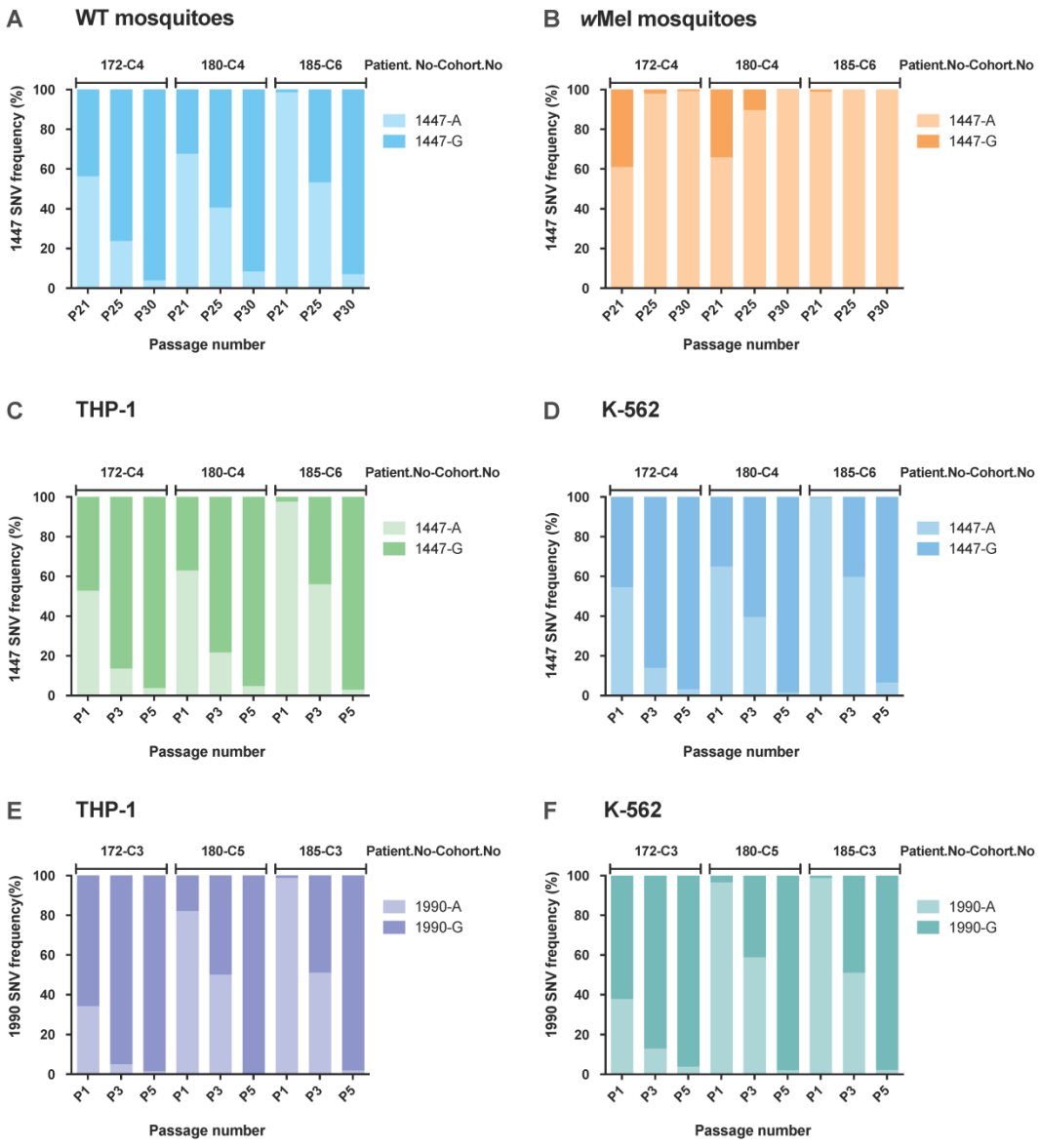

Fig 5: Heatmap of non-synonymous substitutions that developed in the replicate DENV-1 passage experiment.DENV-1 was injected intrathoracically into mosquitoes to establish passage 0 (P0) in three mosquito cohorts per line. The three cohorts were passaged separately until P20 and DENV genomes sequenced to track evolution. SNV frequency is presented for non-synonymous substitutions only, showing only those that increased temporally with passage. Amino acid substitutions within the specified DENV protein are shown on the x-axis for each passage number (P0, 1, 10, 15 and 20), mosquito cohort (1, 2, 3, 4, 5, 6) and mosquito line (*w*Mel-orange and uninfected-blue). Each viral protein is annotated by colour (C: black, E: light green, NS4B: purple, and NS5: khaki).The heatmap was produced with the ComplexHeatmap package in RStudio.